SOLUBILIZATION AND TRANSMEMBRANE DIFFUSION OF N-METHYLQUINOLINIUM ACROSS DIHEXADECYLPHOSPHATE VESICLES

BY

SERVANDO MUÑOZ

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This work is dedicated to my mother, Consuelo Estela, for her unfailing love and support. She once, upon hearing that "light is the shadow of God," taught me that the rainbow must be His signature.

This dedication also recalls the memory of my father, Servando Muñoz, the gentle, often reticent, man who gave her to me in the first place.

"Lovely Lady, dressed in blue, teach me how to pray.
God was just your little boy, and you know the way."

Taken from: "Merry Mary Meditations" by
B. McCarver Snyder (Liguori Publications, 1987).

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Abstract of the Dissertation Presented to the Graduate School
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By

Servando Muñoz

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An investigation is presented concerning the solubilization sites and the dynamics of transmembrane diffusion of the fluorescent biheterocyclic cation N-methylquinolinium across the membrane of single bilayer, gel-chromatographed, dihexadecylphosphate vesicles in 0.0100 M phosphate buffer at pH = 7.45.

Dynamic fluorometry of the gel-chromatographed vesicles containing N-methylquinolinium indicates the presence of a set of excited singlet states which decay obeying biexponential kinetics. The bimodal distribution of the radiative relaxation constants is interpreted in terms of a microheterogeneous distribution of the fluorophore along the endovesicular water-hydrocarbon interface to include two distinct solubilization sites: a longitudinal, or interfacial, binding environment and a transversal or intercalated binding domain.

On prolonged incubation between 60 to 100 hours, at 45°C, it is observed that the fluorescent cations become distributed between the endo- and exovesicular surfaces. Further, the rate of transmembrane diffusion of N-methylquinolinium is determined by the antiparallel flow of charge-dense, fully hydrated, exovesicular cations such as sodium.

The countertransport of fully hydrated sodium ions includes a collection of exponential rate constants that is described in terms of a kinetic model proposed by W. J. Albery and co-workers which invokes the presence of a gaussian distribution with respect to the free energy of activation for transmembrane diffusion. The model requires the use of two adjustable parameters: the dispersive rate constant, k, derived from the mean free energy of activation, and the gaussian width parameter, γ, that is proportional to the standard deviation of the energy barrier. The distribution in the exponential rate constant for the countertransport of charge-dense, hydrophilic, cations is assigned predominantly to the presence of thermally-induced fluctuations in the membranogenic dielectric constant which result from percolation of the hydrocarbon bilayer by water molecules.

Moreover, in the presence of excess external sodium or tetramethylammonium, a drastic acceleration in the rate of discharge of N-methylquinolinium is observed. As the exovesicular fluorescent probes are exchanged and displaced completely into the external aqueous phase, their hyperpolarized diffusion potential triggers an electrostrictive gate that stimulates strongly the countertransport of charge-dense, hydrophilic, cations.

CHAPTER I
INTRODUCTION

In his Hydriotaphia which became public in 1658, Thomas Browne,[1] a country physician and natural philosopher, wrote that "Life is a pure flame and we live by an invisible Sun within us." Robertson[2] has pointed out that this fundamental insight invokes the principle on which all life, as we know it, depends, namely, the transducibility of energy and the ability of living organisms to accumulate different forms of work.

To store, or isolate, energy in its different states, living organisms must be able to provide a constant internal microenvironment that is independent of the changing properties of the bulk macroenvironment in which they exist.[3] Modern living cells implement this function because they are protected by means of a semipermeable barrier or cell membrane.[3]

The membrane of living cells is composed of 50 to 60% proteins and 50 to 40% phospholipids and glycolipids.[4] The latter compounds contain a hydrocarbon double chain attached to a polar headgroup, thereby giving the molecules their amphiphilic nature. The basic morphology of the cell membrane has been described by Singer and Nicolson[5] as a fluid two-dimensional mosaic of phospholipid and glycolipid molecules[6] arranged in the form of a bilayer with the hydrophilic anchors directed away from each other to shield the hydrophobic double chains from the aqueous medium, as indicated in

Figure 1. Furthermore, the structure is held together by the sensitive balance between repulsive interactions, among the polar headgroups, and the attractive van der Waals forces, between adjacent hydrocarbon chains.[7] In addition, an important driving force for the formation of the bilayer skeleton is, certainly, the hydrophobic effect,[8] that is, the increase in entropy that obtains whenever water molecules are displaced from the semi-ordered interior of an aqueous medium.

While the phospholipid and glycolipid molecules make up the skeleton for the cell membrane, the different proteins supported by the matrix regulate the ionic permeability of the hydrophobic barrier by providing ion carriers and channels which promote the storage of energy in the form of electrochemical gradients of cations, especially sodium, potassium, and protons.[3]

The characteristic topology of lipid bilayers is of fundamental importance in controlling the interchange of energy for vital membranogenic processes such as the transmission of electrical impulses along nerve cells, respiration, or the light-driven synthesis of the highly reducing phosphate-containing compound NADPH, dihydronicotinamide adenine dinucleotide, that takes place on the outer surface of the thilakoid vesicles, in chloroplasts.[9,10]

At the molecular level, the interchange of energy depends on the solubilization and the transport of charge across water-hydrocarbon interfaces and closed lipid bilayers. For example, the thilakoids in the chloroplasts of green plants contain a closed bilayer membrane which is made up of glycolipids and proteins, and serves to protect

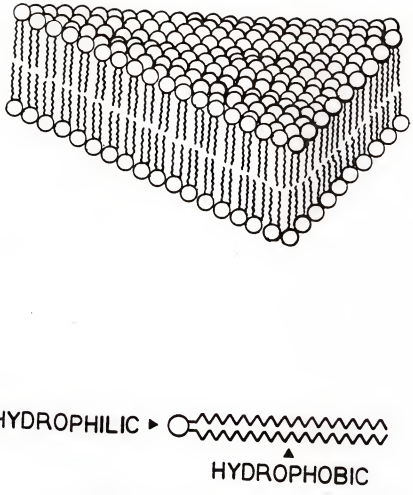


Figure 1. The double layer structure of the membranogenic mosaic.[4]

an internal pocket of water from the main bulk of the intracellular fluid. Accordingly, the topography of the thilakoid vesicle includes at least five distinct binding environments that are necessary for the transduction and storage of radiant energy.[10]

The thilakoid vesicle is suspended in the main bulk of the intracellular fluid to permit the diffusion of NADPH and ATP away from the surface of the organized assembly. Further, on the outer periphery of the thilakoid there exists an anionic hydrophobic-hydrophilic interface where the polar headgroups of the glycolipid molecules are anchored, in addition to $NADP^+$ and a light-driven unit generally represented as photosystem I. Still further within the structure of the thilakoid vesicle,[10] there exists a hydrophobic bind ing environment that extends 70 angstroms into the body of the lipid bilayer and accommodates an additional light-driven unit generally identified as photosystem II. Similarly, within the hydrophobic space reside plastoquinone molecules which are freely soluble in the bilayer matrix, because of their long lipophilic tail,[2,9] and serve as electron relays between the two photosystems.

Proceeding across the hydrophobic bilayer, toward the interior of the organized assembly the topography of the thilakoid vesicle changes again to include another, negatively charged, hydrophobic-hydrophilic interface.[10] Anchored at this interface are the polar headgroups of additional glycolipid molecules, and, very likely, a manganese-containing complex responsible for the evolution of dioxygen.[10] Beyond the internal water-hydrocarbon reef, there extends a pool of water that is shaped in the form of a disk with a transversal

length between 100 and 200 angstroms and a longitudinal axis measuring about 5000 angstroms. The internal pocket of water serves to maintain an electrochemical gradient of protons that arises simultaneously upon illumination with red light.[9,10]

Thus, visible light, which Thomas Browne[11] thought to represent the "shadow of God," triggers the oxidation of the photocell adsorbed on the outer periphery of the thilakoid vesicle to subsequently reduce the neighboring nicotinamide adenine dinucleotide, $NADP^+$, and generate NADPH. Similarly, red light excites the photocell held within the hydrophobic matrix of the bilayer to release electrons that are transported molecularly by the plastoquinone anion, across the bilayer, toward the external hydrophobic-hydrophilic interface. There, the plastoquinone anion sticks its charged headgroup[2] into the aqueous phase and becomes protonated. The resulting plastohydroquinone then participates in the transmembrane transport of hydrogen ions to generate an electrochemical proton gradient by increasing the acidity of the internal pool of water.[2]

Although the details of the transport are not completely understood, the plastohydroquinone is intercepted and oxidized by photosystem I while pumping hydrogen ions across the thylakoid membrane.[9] Moreover, the electrochemical gradient of protons that arises in this manner is subsequently discharged by adenosine triphosphate synthetase, to drive the production of ATP on the exterior surface of the thilakoid assembly. Finally, to complete the photosynthetic cycle, the cationic vacancy that remains in photocell II is filled with electrons extracted from the oxidation of water. Thereupon, with the

assistance of the manganese-containing complex, dioxygen is evolved at the internal water-hydrocarbon reef.[9]

In this fashion, NADPH and ATP emerge as the first detectable intermediates in the synthesis of carbohydrates from the simple starting materials of water and carbon dioxide. Thus, in effect, all life on the surface of our pretty Earth depends on the interchange of radiant energy mediated by the molecular transport of charge across the diverse topography of the thilakoid vesicle.[10]

While the thilakoid assembly represents an astonishing feat of interfacial transport engineering, which at the present time we can only admire, the search has been initiated to investigate model synthetic bilayers that will simulate at least the more elementary functions of living cell membranes.

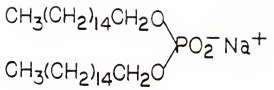
Model Synthetic Vesicles

The first example[11] of the preparation of a closed bimolecular leaflet, or lipid bilayer, came in 1962 when Bangham and Horne found themselves admiring, on the screen of their electron microscope, some multiconcentric lipid assemblies obtained upon suspension of ovolecithin, or egg-phosphatidylcholine, in water. They suggested[12] that the lecithin molecules, when exposed to water, rearrange such that the polar headgroups screen the hydrophobic chains from contact with the aqueous phase. In the molecular assembly thus obtained, the polar lipid molecules are arranged with their lipophilic chains intercalated between the water soluble anchors that point away from each other to generate a bimolecular leaflet, or lipid bilayer. Nevertheless, the water-hydrocarbon interface rapidly becomes curved, as shown in Figure 2, in order to minimize its surface area and therefore its free energy.[12]

In general, a spherical multilamellar structure is obtained with an aqueous layer trapped between adjacent concentric lipid bilayers, as described in Figure 3. As the amphiphiles proceed with their sequential rearrangement, a solute that is initially present in the aqueous medium will similarly penetrate the interlaminar space to remain occluded within adjacent aqueous compartments.[12]

The molecular architecture of the multilamellar vesicle shown in Figure 3, can be simplified by mechanical means, including the use of ultrasonic irradiation.[4] When the multilamellar vesicles are disrupted by means of high frequency sound waves, a suspension of particles with nearly monodisperse radii can be obtained.[4] As Figure 4 illustrates, these particles are much smaller than the multilamellar assemblies and contain an internal pocket of water that is separated from the main bulk of the aqueous solution by a single bilayer of hydrocarbon, hence their name, unilamellar vesicles.

The lipid bilayer of vesicles represents a legitimate model to simulate the functions of living cell membranes because synthetic vesicles possess the two distinctive properties[12] of living cell membranes, as follows: when placed in hypoosmolar solutions, that is, an aqueous medium whose solute concentration is more dilute than that of the medium in which the liposomes are originally prepared, swelling occurs such that the salt concentration for the intravesicular fluid is the same as for the main bulk of the solution. Conversely, when the vesicles are suspended in a hyperosmolar medium, that is, an aqueous system whose solute concentration is greater than that of the



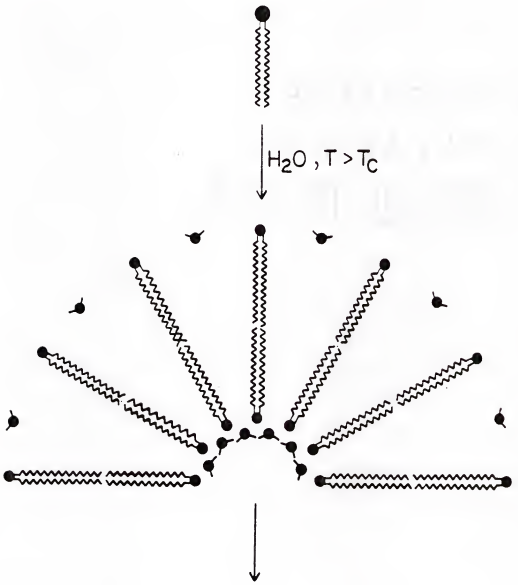


Figure 2. The preparation of liposomes: Tc represents the phase transition temperature of the phospholipid or surfactant compound.[3,12]

Figure 3. The structure of a multilamellar liposome.4,12

Figure 4. The structure of a single bilayer liposome prepared by ultrasonic irradiation of multilamellar vesicles, MLV, or liposomes.

medium in which the vesicles are first prepared, shrinking takes place, as the salt concentration for the intravesicular fluid must increase to match that found in the main bulk of the solution. The shrinking and swelling of liposomes implies the unrestricted movement of water across the lipid bilayer, a property that is typically found with the membranes that protect living cells.

In addition to its osmometric properties, the lipid bilayer experiences thermotropic mesomorphism.[12,13] This cryptic term refers to the property whereby the bilayer exhibits a reversible phase transition that is roughly reminiscent of a melting point.[13,14] Accordingly, the phase transition represents a sharp, narrowly defined, endothermic change in the specific heat of the lipid bilayer. Below the phase transition temperature, the water-insoluble chains in the lipid assembly exist in an extended, all-trans conformation that is usually described as a semicrystalline array, or gel-state. At the phase transition, the hydrophobic membrane becomes completely fluid as the hydrocarbon chains become increasingly disordered because trans-gauche rotations occur in some of the C-C bonds along the chains.[13] However, the entrance into the fluid state must be a cooperative, or concerted process, since the introduction of a single trans-gauche rotation in a C-C bond along the carbon skeleton would cause the terminal methyl group to collide with adjacent hydrocarbon chains.[13]

As a consequence of the introduction of successive trans-gauche rotamers, the effective length of the hydrophobic chains will decrease such that a transversal contraction of the lipid matrix takes place;

to illustrate this event, at the phase transition of lecithin vesicles, there occurs a 20% contraction in the effective length of the membrane.[13]

In addition to the transversal contraction, the introduction of kinks, as the trans-gauche rotameric states are lightly known, causes an increase in the effective volume that the double chains occupy and thereby a lateral expansion of the bilayer.[13] In general, the presence of thermally induced, random contractions and expansions in the lipid bilayer has an important effect on the transport of solutes across the membrane. As the fluctuations occur, local clustering, or domain formation, of the hydrocarbon chains will take place and transient line dislocations, or pores, that may span the entire length of the membrane, will develop.[12] Clearly, these microtunnels are not void but become filled with water molecules arranged in a semicrystalline ice-like configuration.[12]

By means of the transient formation of pores lined with water, the rapid flux of the latter across hydrophobic bilayers is readily explained, and hence, the fact that vesicles behave as ideal osmometers.[12] Moreover, the apparently odd observation concerning the high permeability of the membrane of liposomes towards hydroxide and hydrogen ions when compared, for instance, with metal cations, may also be explained by the presence of such temporal line dislocations[12] through which hydroxide and protons will migrate rapidly according to the well known Grotthus mechanism[15] of proton translocation.

One of the most provocative and important applications of vesicles has been recently introduced with respect to their utility as the carriers of drugs, for therapeutic use, in living organisms.[12] Drawing on the fact that lipid vesicles will occlude solute molecules within their aqueous interior, and that they may be prepared using the same phospholipids and glycolipids from which a target cell is built, it becomes apparent that vesicles can be used effectively to smuggle therapeutic merchandise into the cytoplasm; that is, the intracellular space.[12] In this respect, vesicles or liposomes can interact with living cells in four different ways that include[12]: stable adsorption on the surface of the cells; exchange of phospholipid molecules with the cell membrane; penetration into the cytoplasm as a result of endocytosis--a process whereby vesicles become occluded within identical, although larger, particles made up from lipid material derived from the cell membrane; and finally, their contents may be released into the cytoplasm by the important process of fusion, that is, the lipid material from which the vesicles are prepared becomes incorporated into the cell wall, with the simultaneous intracellular discharge of the compound of interest.[12]

The application of liposomes made up from naturally occurring phospholipids, for energy transduction and storage, or to implement the Trojan horse stratagem, is seriously limited by the structural complexity and degradability of natural phospholipids and glycolipids. Incidentally, the fact that natural phospholipids are structurally complex; optically active; readily degradable; and, as condensation products, can only be prepared in an anhydrous environment,[3] gives

validity to the semisecret belief among bioenergeticists that the genesis of phospholipids in the prebiotic Earth represents the elusive philosopher's stone[16] of the chemical evolutionist.

In order to circumvent the inherent difficulties presented by naturally occurring phospholipids, their synthetic counterparts have been prepared with well-defined structures and improved chemical stability.[17] As an example, synthetic phospholipids can be prepared that contain completely saturated hydrocarbon chains and are, therefore, resistant to air oxidation, especially during ultrasonic irradiation.[17] Simple amphiphilic compounds[4] that form stable closed-bilayer structures include detergent molecules with double aliphatic chains which generally contain between 10 and 18 carbon atoms. Typically, these surfactant molecules are represented by dialkylammonium salts or by diesters of a mineral acid such as phosphoric acid.[4]

Dihexadecylphosphate Vesicles

The synthesis of dihexadecylphosphate, a diester that may be prepared from the condensation of hexadecanol with phosphoric acid, and its subsequent hydration to form closed-bilayer assemblies was initially and simultaneously attempted by Kunitake[18] and Quina,[19] respectively. Quina and coworkers demonstrated that the sonication of dihexadecylphosphate in water, for 15 minutes at 65°C, results in the formation of spherical single-bilayer vesicles having an average diameter of 500 angstroms, as observed by means of transmission electron microscopy. Moreover, their experiments indicate that under the appropriate conditions, the DHP organizate can occlude as much as 10% of the aqueous volume within the intravesicular space.[19]

Perhaps the most important recent application of dihexadecylphosphate liposomes concerns their use by Fendler[20] and his associates to investigate the light-induced electron transfer between the sensitizer dication tris(2,2'-bipyridine)ruthenium(II) and the acceptor methylviologen when they are spatially separated by the surfactant bilayer. In a representative arrangement, the electron acceptor, methylviologen, is adsorbed on the endovesicular surface while the sensitizer is adsorbed on the exovesicular surface. Upon irradiation with visible light, the presence of the cation radical of methylviologen is readily detected. Although the formation of the cation radical might be ascribed to a transmembrane electron transfer, Fendler has suggested that the photoreduction takes place on the outer surface of the liposomes, where the photosensitizer is adsorbed, as a result of transmembrane diffusion of the methylviologen dication.[20]

Furlong[21] and his research associates thereafter pointed out that the use of DHP liposomes to implement transmembrane charge separation in the photosystem designed by Fendler, requires an investigation concerning the ionic permeability of the DHP organizate including the effect of the relative adsorption of cations on the external and internal surfaces; and the effect of the pH of the aqueous medium in which the liposomes are suspended.[21]

Binding Environments

The adsorption sites or, more generally, the different binding environments provided by the matrix of a DHP liposome may be studied

effectively using the mean radiative lifetime of electronically excited molecules. Accordingly, excited states return to the ground state obeying a first order kinetic decay whose rate constant, or its reciprocal value, namely, the apparent radiative lifetime, will be influenced by the local environment observed by the excited state. The resolution of the method to detect various binding domains is, of course, determined by the average duration, or apparent lifetime, of the excited state relative to the process of molecular diffusion. Certainly, fluorescent excited states, with their inherently fast emission, usually satisfy the condition that radiative relaxation to the ground state takes place on a time domain that is narrow when compared with the time evolution of molecular diffusion. Under this condition, the possibility arises that a distribution of fluorescence lifetimes may be observed such that the time-dependent emission of light will not be accurately described with a single first order rate process but rather by means of a sum, or superposition, of exponential terms.[22]

In this respect, Plant,[23] working with liposomes prepared from a mixture of 50% dimyristoylphosphatidylcholine, 40% cholesterol and 10% DHP, has occluded the rapid emitter, xanthene dye, sulforhodamine within the vesicles, and then measured the radiative relaxation of the lowest excited singlet state. For example, at a concentration of intravesicular sulforhodamine equal to 50 millimolar, the simultaneous decay of two distinct emissive states is observed. Moreover, one-third of the decay equation corresponds to an excited state whose

lifetime, at 4.3 nanoseconds, is identical to that of the sulforhodamine in an aqueous environment.[23] Plant, however, did not discuss the biexponential decay function in terms of its potential physical significance.

Similarly, Almgren[24] has observed the temporal fluorescence decay of tris(2,2'-bipyridine)ruthenium(II) occluded within dihexadecylphosphate liposomes, and then detected a bimodal set of distinctive emissive states. In this case, 90% of the decay function corresponds to an excited state whose lifetime, at 490 nanoseconds, is only slightly longer than the 400 nanoseconds measured in an air-saturated, aqueous solution of $Ru(bpy)_2^{+2}$. By means of fluorescence quenching experiments using methylviologen, MV^{+2}, and Cu^{+2} ions as deactivators, Almgren[24] concludes that the $Ru(bpy)_2^{+2}$ is distributed over two binding environments: there exists an ionic exovesicular site at which the adsorbed $Ru(bpy)_2^{+2}$ is quenched efficiently and then there exists a second solubilization site where the fluorophore is protected against quenching with either MV^{+2} or Cu^{+2} ions. Because MV^{+2} and Cu^{+2} are unable to penetrate the liposomes during the time required to perform the quenching experiments, Almgren suggests that the protected residence site corresponds to an intravesicular surface. Furthermore, because of a small bathochromic shift in the fluorescence of $Ru(bpy)_2^{+2}$ with respect to the emission in pure water, Almgren concludes that the protected intravesicular probe is experiencing a hydrophobic environment that requires some contact with the hydrocarbon chains of the surfactant bilayer.

Still, as Almgren[24] appropriately points out, the biexponential decay function may provide only an approximate description of the physical reality underlying the photophysical events. Hence, as Ware[22] has demonstrated, the physical significance of the parameters derived from a binary exponential superposition must be ascertained by the controlled experimental manipulation of the distribution.

Accordingly, to continue the study of the binding environments available in dihexadecylphosphate vesicles, the present work is dedicated, in part, to experiments concerning the fluorescence lifetime of N-methylquinolinium in the presence of the DHP organizate. The effectiveness of N-methylquinolinium in such studies is demonstrated by its highly emissive, nanosecond-lived, lowest excited singlet state which is very sensitive to the polarity, especially the ionic composition, of the medium. Moreover, the molecular skeleton of N-methylquinolinium is comparatively small, as not to introduce a large perturbation in the structure of the liposomes; in particular, the binding sites. Still further, the fluorescence properties of the cation are independent of the pH in the medium, to the extent that the structure does not incorporate acidic or basic functional groups. Finally, the fluorophore is chemically quite stable such that it is not easily degraded with ultrasonic irradiation.

The basic protocol for the photophysical experiments involves the inclusion of the fluorescent probe within the DHP vesicles with the subsequent chromatographic exclusion of the intervesicular N-methylquinolinium. Consequently, the emission from which the apparent

lifetime is measured corresponds to excited states that primarily experience the internal, or intravesicular, binding environments of DHP assemblies.

In analogy with the work of Plant[23] and Almgren,[24] the fluorescence decay of the intravesicular probe is found to be biphasic. An objective of the work presented here, therefore, is to determine the correlation that exists between the microtopography of the organized assembly and the parameters in the fluorescence decay function. To this end, the component lifetimes are compared with the emission lifetime of N-methylquinolinium in media of different composition and ionic strength. Furthermore, quenchers of fluorescence are added to some of the liposome suspensions. The stratagem for the quenching experiments is not complicated: an excited state associated with an aqueous environment, such as the internal pocket of water, or the intervesicular solution, is readily accessible to negatively charged deactivators, like tosylate anion or bromide. Conversely, an excited state associated with anionic sites, such as the negatively charged endovesicular or exovesicular surfaces, is readily accessible to cationic quenchers, like thallium(I).

The change in the size of the vesicles upon osmotic stress[4,12,25] is also useful to continue to probe the physical meaning of the bimodal fluorescence decay. Thus when liposomes are suspended in a hyperosmolar medium, or, conversely, when they are placed in a hypoosmolar one, the corresponding radial contraction, or expansion, and its effect on the intravesicular fluorescence provides knowledge with

respect to the binding sites to which correspond the component lifetimes.

Still another property of the excited state of N-methylquinolinium, which is useful to characterize the residence sites, involves the temperature dependence of the fluorescence. An Arrhenius plot of the natural logarithm of the reciprocal fluorescence lifetime versus the reciprocal absolute temperature is linear, in the range between 278 and 318°K, and provides the respective activation energies of emission. Besides their intrinsic value, these activation energies may be used for comparison with the emission barrier in other media whose properties simulate the binding sites of the intravesicular N-methylquinolinium.

To establish a valid solubilization model, it is also necessary to examine the correlation between the magnitude of the pre-exponential coefficients, in the binary decay equation, and the probability, or mole fraction, of the component fluorescent states. The stratagem in these experiments involves a deliberate and reversible redistribution of the component lifetimes that is implemented by incubating the liposome suspension for increasing periods of time, and then detected by remeasuring the fluorescence decay under ambient conditions. Moreover, the resulting distribution may be reversed to its initial state because, on incubation, the N-methylquinolinium is observed to translocate to the exovesicular surface, where it can be reduced with several aqueous reagents including sodium dithionite.[26]

Dynamics of Electrolyte Translocation

The translocation of electrolytes across a semipermeable membrane generally requires cotransport or countertransport.[27] The simplest form of cotransport is represented by the translocation of a pair of ions having opposite charge. Furthermore, the cotransport may be classified as electrically silent, that is, electrically neutral, or, conversely, as electrogenic.[27] An example of electroneutral cotransport corresponds to the passive diffusion of sodium chloride across a semipermeable membrane, as in a Donnan equilibrium. On the other hand, electrogenic cotransport will take place when the ions exhibit different mobilities, as they diffuse across the membrane proper.[27]

Electrogenic cotransport involves diffusion in the absence of convectional mixing. For example, the movement of hydrochloric acid past a semipermeable membrane; or across the liquid junction between solutions of dissimilar concentration. Since the mobility of the proton is controlled by the modified Grotthus mechanism,[15] which permits the hydrogen ion to move ahead of the counterion flow, a potential difference is created that precludes the further migration of protons across the interface. Because the potential difference that results in this manner is dynamic, it will decay with a rate controlled only by the diffusion of the slower counterion.[27]

Countertransport, on the other hand, involves an exchange of ions with the same sign of charge, by diffusion past an interface. Depending on the relative mobilities of the ions, the countertransport

may be described as electrically silent or, conversely, as electrogenic.[27] In the case when the mobilities of the counter-diffusing ions are greatly different, a temporal transmembrane potential difference arises which will decay gradually at a rate determined by the translocation of the less mobile specie. Accordingly, in biological membranes, electrogenic transport has been likened to the charging process in a capacitor, after a step in the current, followed by the slower decay of the potential difference, determined by the inherent leakage current of the capacitor.[27]

The transient voltage difference that occurs with electrogenic counterdiffusion is usually described as a biionic potential.[28] For instance, Michaelis and Fujita[29] have measured the initial potential difference across negatively charged collodion membranes of high cation selectivity by placing, on opposite sides of the membrane, isotonic solutions of electrolytes with a common anion but dissimilar cations. Michaelis[28] first proposed that the biionic potential is caused by the difference in the membranogenic mobilities of the cations. Sollner,[30] however, emphasized the importance of the relative size, and adsorbability, of the counterdiffusing ions on the magnitude of the biionic potential. On the other hand, the direction in which the membrane is polarized is determined by the more mobile specie; thus, the compartment into which it tends to diffuse will carry a microscopic excess of charge according to the sign of the more mobile ion.[30]

The first observation of a biionic diffusion potential across the pure phospholipid bilayer of liposomes was made by Papahadjopoulos[31] while investigating the self-diffusion of sodium and potassium ions in the presence of anionic phosphatidylglycerol and phosphatidylserine vesicles. Organized assemblies prepared with these phospholipids exhibit the rare property to discriminate between sodium and potassium ions. In this respect, Papahadjopoulos observed that with anionic unilamellar vesicles of phosphatidylserine, and phosphatidylglycerol, the permeability toward potassium is ten times greater than in the case of sodium ions. Furthermore, Papahadjopoulos[31] prepared asymmetric liposomes containing intravesicular potassium chloride with an identical concentration of intervesicular sodium chloride. Upon remeasuring the rate of extrusion of potassium ions, he found that the presence of intervesicular sodium ions inhibits the rate of outward diffusion of potassium ions by 50%.

Papahadjopoulos[31] suggested that the inhibitory effect on the rate of extrusion of potassium by the presence of intervesicular sodium could be explained in terms of a biionic potential generated by the presence of the more permeating potassium cation. Hence, according to the characteristic properties of the biionic diffusion potential, the intervesicular space will carry a microscopic excess of charge of the same sign as the more mobile potassium cation, while an equivalent excess of charge with opposite sign will exist within the vesicles. In addition, the resulting transitory voltage gradient will decay with a rate determined only by the countertransport of the much less

permeating sodium cation; therefore explaining the inhibition in the rate of escape of potassium in the presence of extravesicular sodium ions.[31]

The existence of the biionic potential across asymmetric phosphatidylserine bilayers has been verified by S. Ohki[32] while working with black lipid membranes containing the same concentration of potassium chloride and sodium chloride, respectively, on opposite sides of the membrane. With 50 millimolar KCl in one compartment and 50 mM NaCl in the other, the phosphatidylserine black lipid membrane will generate a transmembrane potential difference of approximately 60 millivolts. Moreover, obeying the properties of a genuine biionic potential, the direction in which the membrane is polarized is determined by the more permeating specie, in this case the potassium cation. Certainly, Ohki[32] found that the compartment containing the sodium chloride showed the higher, that is, the more positive potential. Conversely, the compartment containing the potassium chloride carried the excess of negative charge.

In analogy with the phosphatidylserine and phosphatidylglycerol liposomes, anionic, cation selective, dihexadecylphosphate vesicles containing a high internal concentration of N-methylquinolinium, and a high external concentration of sodium ions, are able to generate the biionic diffusion potential. The polarization of the dihexadecylphosphate membrane follows from the well established fact that charge delocalized ions such as N-methylquinolinium permeate hydrophobic bilayers with a rate, or mobility, that is several orders of magnitude faster than in the case of the much more electrostatically dense sodium cations.[33]

Under the influence of an outwardly directed concentration gradient, the N-methylquinolinium ions tend to diffuse across the membrane of the vesicles, thus establishing a transmembrane potential difference while the static capacitance of the membrane is rapidly charged.[27] However, electrical neutrality requires that the net transport of N-methylquinolinium occurs synchronously with the countertransport of sodium ions. Consequently, the transmembrane voltage[27] must decay at a rate determined by the uptake of the slower permeating sodium. Therefore, it should be possible to utilize the intrinsic fluorescence of N-methylquinolinium as an optical indicator with which to measure the rate of intrusion, or uptake, of sodium ions into dihexadecylphosphate vesicles.

The permeation or translocation of electrolytes, metal cations in particular, across pure phospholipid membranes is an intriguing phenomenon[33] because of the very small, in fact negligible, solubility of hydrated ions in media with a low dielectric constant such as a layer of hydrocarbon.

It is generally accepted[33] that the resolution to this apparent paradox of electrolyte diffusion past the hydrocarbon bilayer is inherently related to the mechanism for the transmembrane transport of water molecules which governs the osmometric properties of biological membranes, and pure phospholipid bilayers; as in the case of liposomes.

H. Trauble[34] first suggested that the transmembrane transport of water in phospholipid bilayers is made possible by the presence of successive trans-gauche rotational conformers along the carbon skeleton. He indicated that the formation of kinks, in the matrix of the bilayer, opens up cavities that become filled with water and thereafter are able to diffuse, as the kinks migrate, across the membrane; Trauble,[34] therefore, regarded kinks as the natural membranogenic carriers of polar solutes. By contrast, using recently acquired data, Nagle[13] has criticized the original work of Trauble by showing that kinks, while numerous, do not represent the dominant rotameric states in the bilayer.

Further knowledge with respect to the structure of the natural carriers in hydrophobic membranes is obtained from the anomalous peak in the self-diffusion of sodium cations at the thermotropic phase transition of pure phospholipid vesicles.[35] As an example, at the phase transition temperature of anionic liposomes made up of dipalmitoylphosphatidylglycerol--a phospholipid molecule with lipophilic double chains that contain 15 carbon atoms--the self-diffusion rate of Na^+ is found to increase about 100-fold. Similarly, neutral liposomes made up of dipalmitoylphosphatidylcholine show an abrupt increase in their permeability to Na^+ at the bilayer phase transition. Immediately after the transition, the self-diffusion rate of Na^+ decreases approximately fourfold with increasing temperature. Consequently, there exists a local maximum in the permeability of sodium ions which arises at the phase transition temperature and extends about a 10 degree range.[36]

The existence of a maximum in the rate at which Na^+ permeates pure phospholipid bilayers is noteworthy especially because on crossing the phase transition of the lipid bilayer, other cations such as potassium and rubidium simply show the expected discontinuous increase in their rate of permeation.[36] Papahadjopoulos[35] and his coworkers attempted to explain the peak in the permeation of Na^+ in terms of enhanced diffusion at the boundaries between liquid and solid domains, or patches, that coexist at the phase transition.

Nagle and Scott[36] retorted that according to the Gibbs phase rule, the coexistence of liquid and solid domains in the presence of excess water, at constant pressure, corresponds to a point discontinuity with respect to the temperature--which is another way to state that the thermotropic phase transition must be very sharp, as would be expected from a cooperative, concerted, event;[13] yet the maximum in the rate of self-diffusion of Na^+ extends over a temperature range of at least 10 degrees.[35] Consequently, the coexistence of liquid and solid domains at the gel to liquid-crystal transition cannot account for the anomalous permeation of Na^+ across pure phospholipid membranes.[36] Alternatively, Nagle and Scott[36] have suggested that the local maximum in the transmembrane diffusion of sodium ions may be explained in terms of lateral density fluctuations in the phospholipid bilayer which proliferate near the transition. These fluctuations act to generate short-lived cavities in the vicinity of the polar headgroups, that is, near the periphery of the organized assemblies. Thus, hydrated ions enter into the transient cavities and subsequently

become trapped as the cavities close. The trapped ions are then in a position to diffuse past the hydrophobic membrane.

An aspect of the mathematical analysis presented by Nagle and Scott[36] which is especially relevant to the work described here concerns the fact that they generalized the model to include an ensemble, or collection, of density fluctuations, implying a corresponding distribution in the size, the volume, and therefore in the aqueous content of the ion-transporting cavities.

In general, the presence of polydisperse membranogenic carriers will cause the first order rate coefficient to be fundamentally different from the unimolecular rate constants normally expected in homogenous media. This fact becomes apparent from the definition of the rate constant for transmembrane diffusion[37]

$$k = P \frac{A}{V_i} \qquad (1)$$

here, the constant P designates the permeability of the membrane, in units of $cm \cdot sec^{-1}$. Furthermore, A represents the surface area of the membrane, while V_i stands for the volume of some microscopic compartment,[37] for instance, the aqueous pocket within liposomes. Hence, it is clear from equation (1) that a distribution in the value of the rate constant may result simultaneously from a distribution in the permeability coefficient, P, of the ion-carrying cavities and from a dispersion in the radius of the vesicles which determines the value of the quotient A/V_i. It is noteworthy, however, that a distribution

in the size, the volume, and thereby in the aqueous content of the membranogenic carriers, has been recognized by Trauble[34] in his original work regarding kink-mediated transport; yet, as it would necessarily simplify his mathematical analysis, he restricted his work to include only a homogeneous, monodisperse, population of carriers.

The influence of dispersive unimolecular kinetics is readily observed during permeation measurements that involve metal cations. In general, the temporal evolution of the process is monoexponential in the early stage of the permeation. However, at longer diffusion times, the dynamic behavior slows down progressively to deviate eventually from the single exponential course. Such behavior has been observed by Phillips[38] and his coworkers while measuring the outward flow of Na^+ from neutral phosphatidylcholine and anionic phosphatidylserine liposomes. Although they attributed the deviation from single exponentiality to the simultaneous release of Na^+ as a result of vesicle fusion events, a similar departure from the first order rate law has been observed in the present work concerning the uptake of sodium ions by anionic dihexadecylphosphate vesicles. Moreover, the strongly fluorescent indicator N-methylquinolinium allows the use of extremely dilute suspensions such that vesicle fusion and additional second order interactions become negligible.

The mathematical analysis of dispersive rates may, for example, be attempted with a sum of exponential terms--as in a biexponential equation.[39] Still, the utility of such an equation is limited to provide an estimate of the rate constant in the early or late stages of the transmembrane diffusion. The knowledge of an approximate initial rate is certainly useful for the purpose of comparison with the permeation rates reported elsewhere in the literature; as the latter generally

correspond to the initial stage of the permeation process of metal cations, while the monoexponential temporal evolution is obeyed.[38]

On the other hand, a more general analysis[39] of dispersive first order dynamics assumes the existence of a gaussian universe, or distribution, with respect to either the rate constant, k, or, alternatively, the natural logarithm of the rate constant, ℓnk. In the latter case, the gaussian corresponds to a distribution in the activation energy.

Albery[39] has suggested that the normal or gaussian distribution in the free energy of activation is more fundamental and probably more likely to be found in nature than an analogous distribution with respect to the rate constant. The basic equation on which Albery has produced his analysis is given by

$$\Delta G^{\neq} = \overline{\Delta G^{\neq}} - \gamma \times RT \qquad (2)$$

here $\overline{\Delta G^{\neq}}$ represents the mean, or average, activation barrier while $\gamma \cdot RT$ signifies the standard deviation, or width, of the gaussian universe. Clearly, the variable x corresponds to the number of standard deviations between an observable free energy of activation and its mean value.

Thus, the parameter γ may take a value greater than or equal to zero, such that its function is to increase the width, or spread, of the gaussian distribution. In this manner, when the value of γ is very small, the spread in the activation energy is quite narrow and the collection may be described as monodisperse. Conversely, as the value of γ increases, the width of the error curve increases proportionately and the collection is termed polydisperse.[39]

As a consequence of the analysis by Albery[39] and his coworkers, it becomes possible to describe the polydisperse, or heterogeneous, collection of unimolecular rate constants as a function of only two parameters: the apparent rate coefficient, $\bar{k}$, derived from the mean free energy of activation $\overline{\Delta G^{\neq}}$, and, of course, the spread, or width, γ, of the mean activation barrier.

The kinetic treatment provided by Albery[39] is particularly useful to investigate the mechanisms from which the dispersion in the rate constant of transmembrane diffusion arises, namely, the radial variation and, as suggested by Trauble[34], the distribution in the permeability coefficient of the cation-carrying cavities.

Accordingly, the width parameter, γ, can be used to demonstrate the presence of both mechanisms. For example, brief sonication times result in suspensions of increasing radial polydispersity.[4] Therefore, at constant temperature, the magnitude of the width parameter, γ, should increase in proportion to the radial heterogeneity of the vesicles. By contrast, the dispersion in the coefficient of permeability for ion-transport is caused by the presence of a heterogeneous distribution of membranogenic carriers. Since the ion-transporting cavities are induced thermally, by lateral density fluctuations in the hydrocarbon bilayer, an increase in the temperature produces a wider range in the size, volume, and aqueous content of the carriers. Therefore, the magnitude of γ should increase in proportion to the temperature, to indicate an increasingly polydisperse mean barrier for cation translocation.

CHAPTER II
BINDING OF N-METHYLQUINOLINIUM IN DIHEXADECYLPHOSPHATE VESICLES

The hydration of dihexadecyl phosphoric acid at 65°C, in a medium buffered with sodium phosphate, at pH = 7.45, produces a very turbid suspension of multilamellar assemblies. The high turbidance of such a heterogeneous suspension, therefore, precludes its use in optical measurements. On the other hand, when the turbid multilamellar particles are irradiated with sound waves of high frequency, or ultrasound, there occurs a gradual decrease in the turbidity and in the size of the aggregates until a blueish, nearly optically transparent suspension of single bilayer liposomes obtains.

When the dimension of the particles is small relative to the wavelength of the incident light, the turbidity, τ, of the medium, that is, the probability per unit length that the stream of light is scattered while traversing the suspension, is given by the Rayleigh equation[40]

$$\tau = \frac{8\pi}{3} \left(\frac{2\pi}{\lambda}\right)^4 \nu\alpha^2 \qquad (3)$$

here ν represents the number of particles per unit volume, λ stands for the wavelength of the scattered radiation and α refers to the polarizability which parallels the cube of the radius of a spherical particle. Further, since the turbidity, τ, represents the extinction coefficient of the incident beam, it is possible to write the statement of the Lambert law[41]

$$\ell n(\frac{I_o}{I}) = \tau\ell \tag{4}$$

in which I represents the intensity of the transmitted light; while I_o represents the intensity of the incident beam, and ℓ is the optical pathlength in the sample. Thus, on combining equations (3) and (4) the result obtains,

$$\frac{1}{\ell} \ell n \ (\frac{I_o}{I}) = \text{constant} \cdot \nu\lambda^{-4} \tag{5}$$

Hence, at constant concentration, and provided the dimension of the aggregates is smaller than the wavelength of the incident light, the turbidance of the suspension will vary linearly with the reciprocal fourth power of the wavelength, to yield an intercept equal to zero.

Barrow and Lentz[42] first applied equation (5) to investigate the uniformity of sonicated egg phosphatidylcholine, or egg lecithin, liposomes in the wavelength range between 650 and 300 nm. Accordingly, in the presence of large, multilamellar, aggregates the graph is nonlinear; in the presence of large unilamellar liposomes, on the other hand, the Rayleigh plot is linear, to yield an intercept which is found to be proportional to the mole fraction of the large single bilayer liposomes in the suspension; finally, in the presence of small unilamellar liposomes, about 500 angstroms in diameter, the linear Rayleigh graph yields an intercept equal to zero. Thus, the method of Barrow and Lentz is useful, in the present work, to determine the uniformity of the sodium dihexadecylphosphate organizates.

The data in Table 1 illustrate the evolution of the turbidance with increasing sonication time for DHP at 65°C and pH = 7.45. For example, with a concentration of DHP equal to 1.83×10^{-3} M, irradiation of the multilamellar dispersion for a brief, less than 1 minute, period of time produces a metastable suspension whose turbidance is observed to be about 0.600, at 500 nm. Further, increasing the sonication time to about 1.5 minutes causes a considerable decrease in the turbidance of the medium, to nearly 0.300, at 500 nm. Finally, sonification beyond 5 minutes generates an optically transparent, blueish, suspension with a turbidance at 500 nm equal to 0.054 ± 0.02. Included in Table 1, as well, are the properties of the Rayleigh graphs of turbidance versus λ^{-4} in the range between 600 and 300 nm for the different sonification times described above. The briefly sonicated, metastable, dispersions produce nonlinear graphs, to indicate the presence of large multilamellar colloids. By contrast, with the longer sonication periods, the colloidal assemblies are observed to obey the Rayleigh equation to generate linear plots of turbidance versus λ^{-4}. Typically, the coefficient of correlation for these graphs is at least equal to 0.9994 and their intercept is small, about 0.0150. The small intercept suggests, in turn, that the mole fraction of large unilamellar vesicles in these suspensions is similarly small.

An inherent limitation of the Rayleigh equation concerns the fact that it does not provide knowledge with respect to the real diameter of the suspended vesicles. In addition, the method cannot be applied in the presence of a chromophore with a high extinction

Table 1. The Turbidance of Unchromatographed Dihexadecylphosphate Vesicles: 1.83 x 10^{-3}M DHP in 0.0100 M NaH_2PO_4, at 25°C and pH = 7.45.

Sonication		Turbidance* at 500 nm	Turbidance versus λ^{-4} 600 - 300 nm	
Time, min	Temp, °C		Intercept	Coefficient of Correlation
<1	65 ± 1	0.582	--	--
1.40		0.325	--	--
5.0		0.0560	0.0150	0.9996
15.0		0.0525	0.0144	0.9994

*Perkin-Elmer 330 spectrophotometer

coefficient in the wavelength range between 600 and 300 nm. Since knowledge of the size, especially the distribution in the diameter of the liposomes, has fundamental importance to understand their physicochemical properties, the use of alternative methods to determine the linear dimensions of the aggregates is necessary. In this respect, the most widely used methods to determine the distribution in the size of liposomes include: transmission and freeze-fracture electron microscopy; as well as dynamic turbidimetry, also known as photon correlation spectroscopy.[43]

With dynamic turbidimetry, an intense beam of monochromatic radiation, or laser, is directed into the colloidal suspension. There, the electric component field of the beam interacts with, and therefore exerts a force upon, the electron clouds of the solvent molecules and the macromolecular aggregates present in the medium. By conventional electromagnetic theory, a charged particle that is accelerated must radiate light. In this case, the radiated light has the same wavelength as the incident beam. Furthermore, because the molecules in the medium are constantly vibrating, rotating and moving about, the intensity of the light radiated at a fixed angle, with respect to the incident beam, will fluctuate in the random fashion of a noise pattern.[43]

Since the macromolecular assemblies are many times more polarizable than the small solvent molecules, the colloidal organizates dominate the intensity of the scattered light. Moreover, because of their large dimension, the macromolecular particles move about slowly and, consequently, the period with which the scattered light fluctuates

is relatively long. Thus, for a small displacement in time, generally in the scale of microseconds, there exists a correlation between consecutive values of the scattered intensity. The extent of the correlation is expressed with a time-dependent equation, generally known as the autocorrelation function. When the suspended particles are uniform with respect to their diameter, a plot of the natural logarithm of the decay of the autocorrelation function versus time is linear; its gradient, or slope, is proportional to the diffusion coefficient of the aggregates. Certainly thereby, the diameter of the particles may be derived from the diffusion coefficient by using the Stokes-Einstein equation.[43]

By contrast, the temporal decay of the autocorrelation equation may be decribed by means of the cumulant technique. This treatment assumes that the transient decay is better simulated as a sum, or superposition, of exponential terms which may arise because there exists a wide distribution in the diameter of the organized assemblies, that is , because the suspension is polydisperse. Accordingly, to determine whether the monoexponential analysis, or the cumulative multiexponential treatment, better simulates the decay of the autocorrelation function, it is necessary to inspect the magnitude of the statistical parameter chi-squared, χ^2.

The chi-squared constant measures the quality with which the calculated parameters simulate the time-dependence of the autocorrelation equation--the better the simulation, the lower the value of chi-squared. In addition to this statistical indicator, in a polydisperse, heterogeneous ensemble of particles, the magnitude of the mean

apparent diameter will vary substantially according to whether the decay is analyzed using the single exponential treatment or, instead, the cumulant technique.

The data presented in Table 2 include the apparent diameter and the chi-squared values obtained from the monoexponential and the second moment, or cumulant, treatments of the dynamic turbidimetric equation for dihexadecylphosphate vesicles. Thus, consider the case of the pure DHP assemblies which yield a zero intercept in the linear Rayleigh graph of turbidance versus λ^{-4}. The chi-squared, 1.33×10^{-7}, derived from the second moment or multiexponential treatment, is 60 times smaller than the χ^2, 8.31×10^{-6}, obtained by applying a single exponential decay to the autocorrelation equation. Furthermore, the apparent mean diameter of 740 angstroms determined with the cumulant simulation is different, outside the experimental margin, from the 860 angstroms determined by assuming a unimodal, or single exponential, distribution in the diameter. Because the apparent diameter is widely different for the two methods, and especially since the second moment treatment provides the better simulation, based upon the smaller chi-squared, it follows that the suspension of pure DHP assemblies is polydisperse.

Nevertheless, the mean particle diameter calculated using the cumulant technique represents the weighted average of the mole fraction, or probability, of the component diameters in the distribution. Alternatively, an effective technique to derive knowledge, from the multiexponential correlation equation, regarding both the magnitude and frequency of the component diameters, involves the construction of

Table 2. The Diameter at 25°C of Dihexadecylphosphate Vesicles Suspended in 0.0100 M NaH_2PO_4, at pH = 7.45,[a] from Dynamic Turbidimetry

				Correlation Function			
				Monoexponential		Second Moment	
		[DHP], M	Recovered DHP	Average Diameter, Å	χ^2	Average Diameter, Å	χ^2
I[b]	Unfiltered	1.83×10^{-4}	--	863	8.31×10^{-6}	740	1.33×10^{-7}
II-A[c]	Gel-filtered[d]	$(4.20 \pm .08) \times 10^{-5}$	9.2±2.0%	1540	5.82×10^{-8}	1500	2.53×10^{-8}
II-B[c]	Gel-filtered[d]	$(4.20 \pm .08) \times 10^{-5}$	9.2±2.0%	1510	1.95×10^{-7}	1470	9.09×10^{-8}

[a] 1.83×10^{-3} M DHP: sonicated 5 minutes at 65°C in 0.0100 M NaH_2PO_4, pH = 7.45.

[b] N-methylquinolinium not occluded.

[c] N-methylquinolinium occluded.

[d] 0.500 mL of the crude suspension is chromatographed using a column of Sephadex G-50, 23.5 cm long x 0.4 cm wide, hydrated with 0.0100 M NaH_2PO_4, pH = 7.45, and eluted using the same buffer system.

a histogram. Thereby, when the multiexponential correlation equation is transformed into a histogram of particle diameters, the data presented in Figure 5 are generated.[44] Clearly, in the suspension of pure DHP vesicles there exists a bimodal distribution with respect to the diameter. The major component of the distribution is the small organizates with a mean diameter about 400 angstroms while the minor component is the larger vesicles which have an average diameter about 1400 angstroms.

By taking the heights of the two maxima in the histogram as indicative of the concentrations of the component diameters, the relative frequency is estimated about 2:1, in favor of the smaller 400 angstrom particles. This result is at variance with the small, near zero, intercept provided by the linear Rayleigh graph of the turbidance versus λ^{-4} which, in turn, suggests that the mole fraction of large unilamellar vesicles in the suspension is very small. Therefore, the relative heights of the two peaks in the histogram do not give the true mole fraction, or probability, of the component diameters in the bimodal ensemble.

To obtain the true mole fraction of the component diameters in the suspension of pure dihexadecylphosphate vesicles, it is necessary to correct the relative frequency ratio for the fact that the larger 1400 angstrom vesicles scatter light much more strongly than the smaller 400 angstrom assemblies. Accordingly, the intensity of the scattered light at some fixed angle, θ, relative to the incident beam is given by[15]

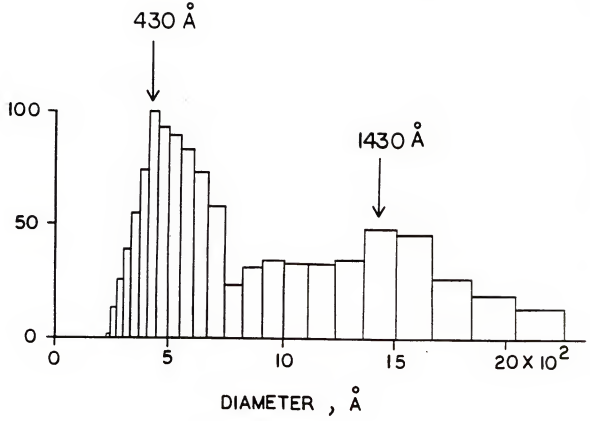


Figure 5. The diameter of pure unchromatographed dihexadecylphosphate vesicles from dynamic turbidimetry at 25°C: 1.83×10^{-4} M DHP in 0.0100 M NaH_2PO_4, at pH = 7.45.

$$I(\theta) = AI_o C_p M(1 + \cos^2\theta) \qquad (6)$$

in which A is a constant that depends on the refractive index of the medium, the wavelength of the incident light, and the distance from the sample cell to the detector; I_o represents the intensity of the incident beam of light, while C_p refers to the concentration of the scattering particles. Finally, M stands for the molar mass of the colloids. Hence, for a given concentration of organizates, at a constant angle of scatter, θ, equation (6) suggests that the intensity of the scattered light will be proportional to the molar mass of the particles.

Therefore, from equation (6), the proportion of light scattered by the large 1400 angstrom organizates, $I(\theta)_\ell$, to the intensity radiated by the small 400 angstrom vesicles, $I(\theta)_s$, is given by

$$\frac{I(\theta)_\ell}{I(\theta)_s} = \frac{\bar{M}_\ell}{\bar{M}_s} = \frac{\bar{N}_\ell}{\bar{N}_s} \qquad (7)$$

where $\bar{M}_\ell$ and $\bar{M}_s$ represent, in turn, the mean molar masses of the large and the small assemblies, while $\bar{N}_\ell$ and $\bar{N}_s$ are the corresponding mean numbers of DHP monomers in each type of vesicles. Moreover, the number $\bar{N}$ of DHP monomers in a vesicle with diameter $\bar{D}$ and bilayer thickness δ is calculated according to the relationship

$$\bar{N} = \frac{4\pi\left[(\bar{D}/2)^2 + (\frac{\bar{D} - 2\delta}{2})^2\right]}{A_{DHP}} \qquad (8)$$

in which A_{DHP} represents the area occupied by the anionic headgroups on the surfaces of the dihexadecylphosphate vesicle.

Using a value of 50 angstroms as the thickness of the bilayer, and 70 $\mathring{A}^2$ as the estimate of A_{DHP} suggested by Hurst[45] and his co-workers, the large 1400 angstrom liposomes observed in the histogram in Figure 4 are estimated to contain 1.6×10^5 DHP gegenions; on the other hand, the smaller, 400 angstrom liposomes are estimated to contain 1.1×10^4 monomers. Consequently, from equation (7)

$$\frac{I(\theta)_{\ell}}{I(\theta)_{s}} = \frac{1.6 \times 10^5}{1.1 \times 10^4} = \frac{15}{1}$$

the large 1400 angstrom vesicles scatter light with an intensity that is approximately 15 times stronger than the small 400 angstrom assemblies.

Using the above estimate of the relative intensities of light scattered by the two assemblies, the apparent 2:1 concentration ratio derived from the peak maxima in the histogram can be corrected to give the true 2:0.07 concentration ratio, in favor of the small 400 angstrom vesicles. Therefore, the mole fraction of the large unilamellar vesicles in the suspension is estimated to be about 3% which is now in qualitative agreement with the small, near zero, intercept obtained from the linear Rayleigh plot of turbidance versus λ^{-4} in the wavelength range between 600 and 300 nm.

It is noteworthy that the preceding calculation is quite analogous to the case of two chromophores which absorb light at different wavelengths, and whose concentration ratio needs to be determined. By normalizing to the same extinction coefficient, that is, by correcting for the difference in the molar absorptivities between

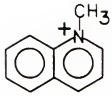
the two chromophores, the concentration ratio may be calculated directly, from the relative height at the maxima of the corresponding absorption bands.

It is also worthy of note that a bimodal distribution of dihexadecylphosphate vesicles has been observed by Hurst[45] and his collaborators at pH = 7.8 in tris(hydroxymethyl)aminomethane. Similarly, Furlong[21] observed a bimodal distribution with DHP vesicles in their acidic form. The minor component in the binary distribution found by Furlong and coworkers represents 1-2% large vesicles with a diameter about 700 angstroms, while the major component represents smaller vesicles with an average diameter about 300 angstroms.

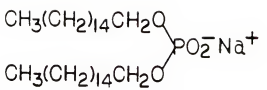
The Inclusion of N-methylquinolinium

The method to occlude, or trap, the fluorescent probe N-methylquinolinium within the dihexadecylphosphate assemblies requires the simultaneous presence of the hydrated, multilamellar, DHP and the fluorophore during the sonication treatment which produces the unilamellar vesicles. After sonification, the vesicles must be separated from the excess, intervesicular N-methylquinolinium. The exclusion of the intervesicular fluorophore may be accomplished by means of a molecular weight filtration using hydrated Sephadex as a gel which restricts the passage of compounds of low molecular mass such as N-methylquinolinium, and, conversely, allows the unrestricted flow of macromolecular particles as in the case of the DHP aggregates, as shown in Figure 6.

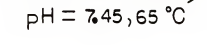
The evolution of the gel-filtration is presented in the chromatogram in Figure 7. From the intrinsic fluorescence of N-methylquinolinium, two component bands are observed during the separation:



AQUEOUS



THIN LAYER



SEPHADEX

G-50

Figure 6. Schematic outline for the inclusion of N-methyl-quinolinium within dihexadecylphosphate vesicles.

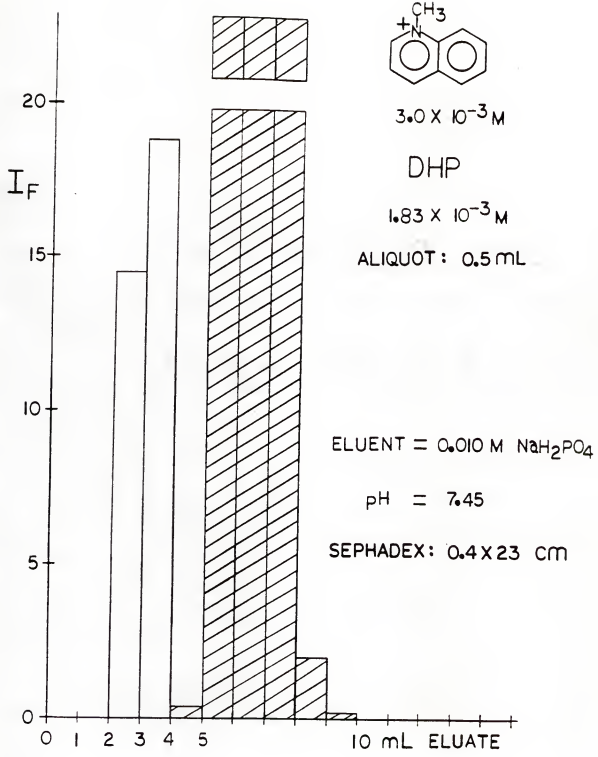


Figure 7. Gel-chromatographic separation of intravesicular N-methylquinolinium.

the weakly flourescent band which elutes with the void volume of the gel-matrix corresponds to the fluorophore occluded, or rather sequestered, within the vesicles; the strongly fluorescent band which elutes afterwards has a retention volume equal to that of the free, aqueous, fluorophore and corresponds thereby to the unoccluded N-methylquinolinium.

The dynamic turbidimetric diameter and the percent of phospholipid recovered from the gel-chromatographic separation are listed, as well, in Table 2. It is significant that the apparent diameter of the gel-chromatographed dihexadecylphosphate vesicles, suspended in phosphate buffer at pH = 7.45, is increased drastically to about 1500 angstroms. It is also found that about 9% of the phospholipid is recovered from the gel-filtration. Because the N-methylquinolinium is a small, flat, cation its presence is unlikely to cause a drastic increase in the diameter of the vesicles. Alternatively, the low percent recovery of the phospholipid suggests that a redistribution of the component diameters has probably taken place such that the more turbid liposomes dominate the light scattering properties of the suspension, thereby causing the large increase in the apparent diameter of the assemblies.

A redistribution of the component diameters of the DHP assemblies upon gel-filtration, with hydrated Sephadex G-50, is evident from electron microscopic inspection of the eluate which indicates the presence of vesicles whose spherical diameter is calculated to be 500 angstroms. Moreover, additional transmission as well as freeze-fracture electron micrographs demonstrate a polydisperse distribution in the diameter of the gel-filtered liposomes.

The polydispersity of the gel-filtered vesicles is evident from the dynamic turbidimetric data in Table 2 corresponding to the suspensions II-A and II-B obtained from duplicate preparations. In the case of suspension II-A the single exponential treatment of the autocorrelation equation yields an apparent diameter about 1500 angstroms with a chi-squared equal to 5.82×10^{-8}. By comparison, the second moment method gives an identical mean diameter of 1500 angstroms and a lower chi-squared equal to 2.53×10^{-8}.

Similarly, in the case of suspension II-B, the monoexponential simulation generated a vesicle diameter about 1500 angstroms and a x^2 equal to 1.95×10^{-7}. By comparison, the second moment data provide the same mean diameter of 1500 angstroms and a lower chi-squared at 9.10×10^{-8}. Although the mean diameter value is independent of the method by which the turbidimetric equation is treated, the magnitude of the chi-squared indicator corresponding to the second moment or cumulant method is consistently lower, by a factor of 2, than the single exponential simulation to indicate that the gel-filtered suspension of vesicles is polydisperse.

Furthermore, the fact that the large unilamellar assemblies represent only a small fraction, about 3%, of the vesicles present in the unfiltered suspension, and yet, they dominate the intensity of the scattered light in the chromatographed eluate, suggests that the large organizates are adsorbed less strongly by the stationary phase than the small vesicles.

Finally, regarding the low percent recovery of DHP, it is noteworthy that the adsorption of phospholipid molecules by hydrated gels, like Sephadex G-50, is well known and may be regarded as an inherent limitation of such separatory matrices.[46]

The Solubilization Sites of N-methylquinolinium

In general, vesicles or liposomes are made up of the seven distinct binding environments described in Figure 8. Thus, from the perspective of a probe molecule in the presence of these assemblies, there is a set of two predominantly aqueous environments which include the main volume of the solution and the internal pocket of water, respectively; likewise, there is a set of two interfacial binding sites, one on the outer periphery of the organizates while the other delineates the boundary of the internal pool of water. These hydrophilic-hydrophobic interfaces create, in turn, a subset of two distinct binding sites: a probe molecule may be adsorbed in a longitudinal or tangential position with respect to the plane of the surface of the organizate or, alternatively, the probe may protrude into the hydrophobic bilayer. The latter, transversal adsorption, is expected to occur particularly with negatively charged vesicles, as in the case of DHP, because of the presence of electrostatic repulsions among the headgroup anchors which generate fissures on the surface of the hydrophobic-hydrophilic interfaces to facilitate intercalation of the solute molecules. At last, in contrast to the interfacial sites, the interior matrix of the hydrocarbon bilayer provides a microenvironment to accommodate the less polar, more hydrophobic probes.

As suggested in the Introduction, the N-methylquinolinium is an effective probe to investigate the topography of dihexadecylphosphate vesicles at pH = 7.45. This fluorophore is chemically stable, to

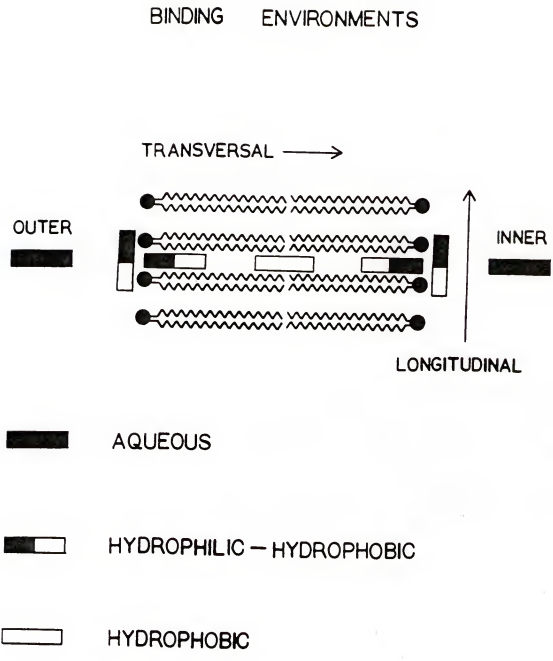


Figure 8. Schematic microtopography across the hydrocarbon bilayer of surfactant vesicles.

resist degradation during ultrasonic irradiation to produce single bilayer liposomes; it is independent of the pH in the medium, to the extent that its structure does not include acidic or basic functional groups; and, most importantly, the N-methylquinolinium has a small, flat, molecular skeleton which is unlikely to modify the topology of its solubilization sites.

In addition to its ideal chemical properties, the N-methylquinolinium has a highly emissive, rapid--nanosecond-lived--lowest excited singlet state whose apparent radiative lifetime is sensitive to the particular microenvironment of the probe. In this regard, the unimodal or monoexponential fluorescence lifetimes of N-methylquinolinium observed in media of different composition are presented in Table 3. For example, in air saturated or degassed water, the fluorescence lifetime is equal to 14.4 nanoseconds; in a buffered medium such as 0.0100 M NaH_2PO_4, at pH = 7.45, the lifetime of N-methylquinolinium is reduced to 8.88 ns; similarly, at pH = 7.45, in 0.200 M sodium dodecylsulfate, the lifetime of the now micellized N-methylquinolinium is 10.1 nanoseconds.

Accordingly, the presence of fluorescence deactivators reduces the radiative time constant while obeying Stern-Volmer dynamics. For instance, in Table 3, the Stern-Volmer quenching constants, K_{sv}, for the anionic deactivators toluenesulfonate and bromide are respectively equal to 44.7 and 135 M^{-1}. Thus, in the presence of 0.0100 M sodium bromide, the lifetime becomes 3.80 nanoseconds.

Conversely, perhaps the only readily available cationic deactivator which does not give an insoluble precipitate in solution of sodium phosphate, at pH = 7.45, is thallium(I). As listed in

Table 3. The Fluorescence Lifetime of N-methylquinolinium[a] in Aqueous Media of Different Composition at 25°C.

	Buffer	pH	K_{sv}, M^{-1}	τ, ns	χ^2
Distilled water	--	7.00	--	14.4	0.909
--	0.0100 M NaH_2PO_4	7.45[b]	--	8.88	1.00
0.0500 M $NaClO_4$	0.0100 M NaH_2PO_4	7.45[b]	--	10.6	1.21
0.200 M SDS	0.0100 M NaH_2PO_4	7.45[b]	--	10.1	1.21
0.0100 M NaBr	0.0100 M NaH_2PO_4	7.45[b]	135[c]	3.80	--
0.0100 M NaTsO	0.0100 M NaH_2PO_4	7.45[b]	44.7[d]	6.10	--
0.0100 M $T\&NO_3$	0.0100 M NaH_2PO_4	7.45[b]	15.7[e]	7.70	--

[a]1 μM N-methylquinolinium perchlorate added.
[b]About 0.64 equivalents of NaOH is added to generate a pH = 7.45.
[c]In the range between 3.00 and 27.1 mM added NaBr, the coefficient of correlation is 0.99997 and the intercept is equal to 0.964.
[d]In the range between 3.00 and 20.0 mM added sodium toluenesulfonate; the coefficient of correlation is 0.9987 and the intercept is equal to 1.108.
[e]In the range between 1.50 and 22.5 mM added $T\&NO_3$, the coefficient of correlation is 0.997 and the intercept is equal to 0.973.

Table 3, the Stern-Volmer quenching constant for this cationic deactivator is 15.7 M^{-1}. Thus, in the presence of 0.0100 M $T\ell NO_3$, the emission time constant is 7.70 nanoseconds. Incidentally, the fact that thallium(I) is the least efficient deactivator in the set--as evidenced from its comparatively small value of K_{SV}--is probably a consequence of unfavorable electrostatic repulsions with the cationic fluorophore.

Concerning the transient decay, I(t), of N-methylquinolinium associated with the dihexadecylphosphate vesicles in 0.0100 M NaH_2PO_4, at pH = 7.45, it is adequately described by means of a biexponential, four-parameter, superposition equation

$$I(t) = A_\ell e^{-t/\tau_\ell} + A_t e^{-t/\tau_t} \qquad (9)$$

where $1/\tau_\ell$ and $1/\tau_t$ stand for the corresponding radiative rate constants or reciprocal lifetimes for the component singlet excited states of the fluorophore. A_ℓ and A_t are the pre-exponential coefficients, or statistical-weight constants, whose magnitude may represent the mole fractions of the lifetimes in the binary set. Certainly, the fidelity with which the calculated parameters τ_ℓ, τ_t, A_ℓ and A_t simulate the temporal decay of the fluorescence, is indicated by the chi-squared constant, whose definition is somewhat different from the reduced chi-squared calculated, for example, in dynamic turbidimetry. Typically, an adequate simulation of the fluorescence decay yields a value of chi-squared less than or equal to 1.30.

The N-methylquinolinium which is associated with the dihexadecylphosphate vesicles, has the bimodal temporal evolution of

fluorescence, at 25°C, recorded in Table 4. The component fluorescence time constants T_ℓ and T_t are, respectively, 9.70 nanoseconds and 3.80 ns. Likewise, the corresponding pre-exponential factors A_ℓ and A_t are, in turn, determined to be 0.24 and 0.76 while the chi-squared is 1.13. Moreover, it should be pointed out that the optical density in the chromatographed suspension of vesicles is maintained about 0.0600 or less. Hence, excitation at an isosbestic point, to normalize for the difference in the extinction coefficient of the intravesicular fluorophores, is not necessary. Accordingly, the parameters derived from equation (9) should be interpreted in the light of a solubilization model that defines the component lifetimes and corresponding statistical frequencies in terms of their most probable binding environment.

Collisional Deactivation Method

The use of charged collisional deactivators like bromide, toluenesulfonate and thallium(I) provides an effective method to investigate the binding sites of the N-methylquinolinium fluorophore in the presence of anionic dihexadecylphosphate vesicles. Accordingly, the fluorophore that resides in an aqueous microenvironment, secluded from the negatively charged surfaces, will be readily accessible to the bromide and tosylate quenchers. Conversely, when the probe is adsorbed on the anionic surfaces, efficient deactivation will take place by means of the cationic quencher thallium(I).

Endovesicular adsorption.-- Consider, therefore, the temporal fluorescence of N-methylquinolinium in the gel-chromatographed

Table 4. The Emission Lifetime of N-methylquinolinium in Gel-chromatographed[a] Dihexadecylphosphate Vesicles and in the Presence of Fluorescence Deactivators at 25°C.

Deactivator		τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^{2b}
--	--	9.67	24	3.79	76	1.13
0.0271 M NaBr	intervesicular	9.64	20	3.73	80	1.28
0.00700 M NaTsO	intravesicular[c]	7.54	20	3.46	80	1.16

[a]In 0.0100 M NaH_2PO_4 at pH = 7.45. The conditions for the gel-chromatographic filtration are described in Table 2.

[b]The lamp calibration curve, or instrument response function, is displaced two channels to the left of the sample decay curve, to compensate for the wavelength-dependent residence time of the signal in the photomultiplier tube, and thus to minimize the value of chi-squared.

[c]The vesicles are prepared by sonication in the presence of 7.00 millimolar N-methylquinolinium toluenesulfonate and gel-chromatographed subsequently to exclude the intervesicular fluorophore.

dihexadecylphosphate vesicles at pH = 7.45. When 0.0270 M NaBr is added to the fluorescent gel-filtered assemblies, there is little change with respect to the component time constants of emission, as demonstrated by the data in Table 4. By comparison, from Table 3, it is calculated that in homogeneous solution, the addition of 0.0270 M NaBr deactivates the lifetime of the fluorophore from 8.88 to 1.88 nanoseconds. Consequently, the fluorescent emission of the probe, in the chromatographed organizates, arises predominantly from an environment that is inaccessible to the bromide deactivator, quite probably the interior of the vesicles.

In an alternative experiment, the anionic quencher toluenesulfonate may be placed within the interior of the vesicles. Thus, the lowermost entry in Table 4 corresponds to the transient fluorescence arising from the gel-chromatographed eluate of dihexadecylphosphate vesicles prepared in 0.0100 M NaH_2PO_4, at pH = 7.45, and in the presence of 7.00 millimolar N-methylquinolinium toluenesulfonate. The value of τ_ℓ becomes 7.50 nanoseconds, which is about two nanoseconds lower than the corresponding long-lived component observed when the vesicles are prepared in the presence of 10 millimolar N-methylquinolinium perchlorate. Because the hydrophilic, anionic, toluenesulfonate quencher is probably associated with the aqueous interior of the vesicles, the preceding observation suggests that a fraction of the occluded fluorophore is adsorbed by an environment where substantial contact with water molecules takes place such that the binding sites are accesible to the toluenesulfonate deactivator. By contrast, the short-lived time constant, τ_t, remains the same, within the experimental margin, to indicate that its corresponding binding sites are protected well against deactivation by toluenesulfonate.

A comparison of the bimodal time constants of emission for the occluded N-methylquinolinium with the unimodal lifetimes recorded in Table 3 indicates, however, that τ_ℓ is similar to the single exponential lifetime, 10.1 nanoseconds, of N-methylquinolinium in the presence of 0.200 M sodium dodecylsulfate at pH = 7.45. Now, because the microenvironment provided to a hydrophobic solute, such as N-methylquinolinium, by an SDS micelle corresponds to a water-hydrocarbon interface, and since Zoltewicz and Muñoz[47] have recently demonstrated that N-methylquinolinium is completely micellized beyond 0.100 M SDS, it is clear that the lifetime τ_ℓ, at 9.70 nanoseconds, may well correspond to the water-hydrocarbon reef available at the periphery of the internal pocket of water within the dihexadecylphosphate vesicles.

To continue to characterize the bimodal fluorescence decay of the intravesicular probes, it becomes necessary to prepare a detailed inventory of the composition of the intravesicular medium. In general, the intravesicular concentration of the solutes which are initially present in the hydrating medium is determined by their binding affinity towards the incipient anionic hydrophilic-hydrophobic surfaces of the vesicles.

Consider, therefore, the case of anionic and cationic solutes. For the solubilization of anionic matter, its intravesicular concentration is expected to be lower than in the bulk hydrating medium because the degree of dissociation of the monomer amphipaths, or gegenions, on the surfaces of incipient vesicles may be comparatively high, such that some of the anionic solute is excluded from the interior of the assemblies. As an example, in the present work, the

intravesicular concentration, $[TsO^-]_i$, of toluenesulfonate may be estimated by using the Stern-Volmer relationship

$$\frac{\tau_o}{\tau_\ell} = 1 + K_{sv}[TsO^-]_i \qquad (10)$$

where from the data in Table 3, the lifetime, τ_o, of the N-methylquinolinium in the absence of toluenesulfonate, at pH = 7.45, is 8.88 nanoseconds, while the Stern-Volmer quenching constant, K_{sv}, is 44.7 M^{-1}. Furthermore, from Table 4, on setting τ_ℓ equal to 7.50 ns, the intravesicular concentration of toluenesulfonate is readily calculated about 4.0 millimolar. By comparison, the concentration of toluenesulfonate in the hydrating medium in which the vesicles are prepared is 7.0 millimolar. Therefore, a fraction of the anionic toluenesulfonate has probably been excluded from the intravesicular volume, perhaps as the result of unfavorable electrostatic repulsions with the anionic surfaces of the incipient vesicles. In this respect, Hurst[45] and his associates have observed similarly that ferricyanide, $Fe(CN)_6^{-3}$, is in part excluded from the interior of the dihexadecylphosphate vesicles prepared in tris(hydroxymethyl)aminomethane at pH = 7.8.

The preceding analysis indicates that the intravesicular concentration of other anions, in particular mono- and dihydrogen phosphate, will be comparatively low in respect to their concentration in the intervesicular medium. This observation might account for the fact that the fluorescence lifetime, τ_ℓ, is slightly longer than the emission time constant of the fluorophore in the pure phosphate buffer

because the phosphate anions can deactivate the fluorescence of N-methylquinolinium. For instance, when 50 millimolar sodium perchlorate is added to the phosphate buffer, the time constant of N-methylquinolinium increases to 10.6 nanoseconds; therefore, upon replacing the phosphate salts in the buffered medium with an equivalent amount of sodium perchlorate, the radiative time constant will increase to at least 14.4 nanoseconds--which is the lifetime of N-methylquinolinium in pure water--and probably longer because the presence of the sodium perchlorate induces a corresponding increase in the fluorescence lifetime.

Regarding the adsorption of cationic matter, the phosphate buffer in which the DHP assemblies are prepared contains two cationic species whose properties are widely different, namely, sodium and N-methylquinolinium ions. Since approximately 0.64 equivalents of sodium hydroxide must be added to 0.0100 M NaH_2PO_4 to raise its pH to 7.45, the concentration of sodium cations in the hydrating buffer is about 20 millimolar. In comparison, the concentration of N-methylquinolinium is equal to 10 millimolar, while the amount of DHP present in the medium corresponds to 6.5 millimolar.

Thus, as the dihexadecylphosphate monomers undergo their sequential rearrangements, ion-pair formation with either sodium or N-methylquinolinium as the counterions will take place. In this regard, the interaction of sodium ions with the anionic DHP amphipaths is purely electrostatic; by contrast, the association of N-methylquinolinium with the anionic water-hydrocarbon interfaces of incipient vesicles

is determined by electrostatic and, more importantly, by hydrophobic forces. Moreover, Zoltewicz and Muñoz[47] have also demonstrated that in contact with the water-hydrocarbon interfaces presented by sodium dodecylsulfate micelles, the binding constant of N-methylquinolinium is nearly 50 times greater than the typical, purely electrostatic association constant of hydrophilic, univalent, metal cations. Hence, as the relative concentrations of N-methylquinolinium and sodium cations in the hydrating medium are comparable, and particularly since the hydrophobic binding of the former is much stronger than the purely electrostatic association of sodium, it follows that the dihexadecylphosphate gegenions will ion-pair predominantly with the biheterocyclic cations.

From the fact that the anionic DHP monomers are paired predominantly with the hydrophobic N-methylquinolinium ions, it is possible to obtain an estimate of the intravesicular concentration of the fluorophore which, in turn, can be used to derive knowledge concerning the distribution of the probe among the solubilization sites within the organizates. For instance, from equation (8), by setting A_{DHP} equal to 70 angstroms squared, as recommended by Hurst,[45] a vesicle with a diameter about 1400 angstroms is calculated to contain approximately 7.6×10^4 DHP monomers on its internal, inwardly-facing, monolayer. Electroneutrality, therefore, requires the presence of an equivalent amount of N-methylquinolinium cations in the intravesicular space. Now, because the volume of the intravesicular space can be calculated from the internal radius of 650 angstroms, obtained by assuming a hydrophobic bilayer length about 50 angstroms, it is clear that the internal concentration of the fluorophore is given by

$$[FL]_i = \frac{7.6 \times 10^4 / N_A}{\frac{4\pi}{3} (650 \times 10^{-8} \text{ cm})^3 \times \frac{1L}{1000 \text{ cm}^3}} = 0.10 \text{ M}$$

where N_A represents Avogadro's number.

Furthermore, because the sodium cations do not ion-pair to a significant extent with the DHP monomer anions, the intravesicular concentration of Na^+ will be approximately 0.0200 M which is the same as in the hydrating, buffered medium. Consequently, the 1400 angstrom vesicles contain about 14000 sodium cations in their aqueous pockets.

On the other hand, a vesicle with a diameter about 400 angstroms is estimated to contain about 4.0×10^3 N-methylquinolinium ions from which their internal concentration may be estimated about 0.50 M. Again, because of the small tendency of the DHP monomer anions to ion-pair with the sodium cations, that is, in the presence of a comparable concentration of the hydrophobic N-methylquinolinium ions, the intravesicular concentration of Na^+ will be about 0.0200 M or the same as in the hydrating buffer. Hence, the 400 angstrom vesicles will contain about 170 sodium cations in their aqueous pockets.

It is clear that by virtue of its hydrophobic nature, as well as because it represents the predominant intravesicular cationic species, the N-methylquinolinium exists in close association with the inwardly facing, internal hydrophobic-hydrophilic interface. In the case of the 400 angstrom vesicles, the problem becomes to accommodate, or to bind, 4000 hydrophobic N-methylquinolinium ions on the endovesicular water-hydrocarbon reef.

According to Figure 8, the endovesicular surface contains a set of two distinct binding sites in which lateral and intercalated adsorption are feasible, with respect to the plane of the surface. In the case of lateral, or longitudinal, adsorption the endovesicular surface becomes coated with a monolayer of N-methylquinolinium ions. Therefore, in order to calculate the maximum number of fluorescent probes that are adsorbed laterally, it is necessary to know the total area of the inner surface as well as the area occupied by a single N-methylquinolinium adsorbate.

It is possible to obtain an estimate of the area per adsorbed N-methylquinolinium fluorophore from the work of Furlong[21] and his collaborators concerning the surface-saturation of unilamellar DHP vesicles, at pH = 8.0, with a monolayer of methylviologen cations. They report that when the DHP assemblies are coated with a monolayer of methylviologen, each cation takes up an area about 185 angstroms squared. Hence, as the methylviologen skeleton is geometrically equivalent to the N-methylquinolinium, that is, both ions are bihexagonal and on a flat surface they will occupy very nearly the same area, it follows that a working estimate of the area occupied by the N-methylquinolinium ion is about 200 angstroms squared.

By using this estimate, the mole fraction, F_{ℓ} , of the N-methylquinolinium fluorophore, adsorbed as a thin layer on the endovesicular surface of the small vesicles whose internal radius is 150 angstroms, becomes

$$F_{\ell} = \frac{4\pi\ (1.5 \times 10^2\ \text{Å})^2}{4.0 \times 10^3\ \text{cations} \times \underset{\text{cation}}{2.0 \times 10^2\ \text{Å}^2}} = 0.35$$

Thereby, the area provided by the endovesicular surface is sufficient to accommodate 35% of the total number of fluorescent cations which are ion-paired with the inwardly-facing dihexadecylphosphate gegenions. However, it is significant that the fraction of N-methylquinolinium counterions which is adsorbed longitudinally by the endovesicular DHP surface is independent of the diameter of the organizates. In general, for a vesicle with an internal radius, r_i, whose monomeric headgroups occupy the area, A_{DHP}, the preceding calculation may be rewritten in the following manner

$$F_{\ell} = \frac{4\pi(r_i)^2}{\underset{A_{DHP}}{\frac{4\pi(r_i)^2}{}}\ \text{cations} \times \underset{\text{cation}}{2.0 \times 10^2\ \text{Å}^2}} = \frac{A_{DHP}}{2.0 \times 10^2\ \text{Å}^2}$$

where the quotient $4\pi(r_i)^2/A_{DHP}$ gives the number of anionic monomers on the inner-surface and hence the number of occluded N-methylquinolinium counterions. Clearly, then, the fraction of the fluorescent probes which is adsorbed longitudinally is determined only by the ratio of the area occupied by the anionic headgroup to that occupied by the fluorophore. In the present case, the stoichiometry corresponds to about three DHP monomer headgroups for each lateral, or longitudinal, N-methylquinolinium adsorbate.

Hereafter, the problem becomes to accommodate the remaining 65% of the fluorescent probes on the endovesicular surface. In this respect, the transversal, more hydrophobic, sites which arise along the inner surface provide appropriate fissures into which the hydrophobic N-methylquinolinium probes can intercalate.

The existence of these fissures which result from unfavorable electrostatic repulsions among the anionic headgroups, or anchors, is also apparent from the work of Furlong[21] and his associates on the saturation of DHP vesicles with a monolayer of methylviologen dications, at pH = 8.0. For example, in order to calculate the area per adsorbed methylviologen, they utilized a value of 35-40 angstroms squared for the surface occupied by the dihexadecylphosphate anionic headgroup, as estimated from molecular models. Still, the area of the methylviologen dication that they derived thus, does not correspond with the value measured independently from its adsorption on the surface of inorganic oxides of well known specific areas such as SiO_2, RuO_2 and TiO_2.

On account of the intermonomeric electrostatic repulsions which develop particularly at neutral or basic pH, Hurst suggested that Furlong and his coworkers raise the surface per anionic DHP headgroup to 70 angstroms squared. Thereupon they recalculated the area per adsorbed methylviologen to yield 185 angstroms squared, a value that corresponds with that determined from adsorption measurements on inorganic oxides.

Hence, the packing density of the anionic dihexadecylphosphate amphipaths on the surfaces of the vesicles is comparatively low

because, as suggested above, the intermonomeric electrostatic repulsions generate fissures along the endovesicular surface where the flat, hydrophobic, N-methylquinolinium luminophores can intercalate, probably without altering the diameter of the organized assemblies. Thus, the remaining 65% of the total occluded N-methylquinolinium will experience transversal adsorption on the hydrophobic-hydrophilic endovesicular interface.

In Table 5 is recorded a set of preexponential coefficients, which is representative of the precision in the values of A_ℓ and A_t encountered while measuring many fluorescence decays. It is significant that the calculated mole fractions, F_ℓ and F_t, for the longitudinal and transversal endovesicular adsorption of N-methylquinolinium, respectively 35% and 65%, correspond within the natural uncertainty with the average preexponential coefficients, A_ℓ and A_t, observed by experiment, in turn 30 ± 4% and 70 ± 4%.

Furthermore, consider the photophysical properties of the N-methylquinolinium intercalated among the nonpolar hydrocarbon chains of the surfactant monomers. In the event that on proceeding to the excited state the polarity of N-methylquinolinium should decrease, then a hydrocarbon environment would be expected to enhance the radiative lifetime. The converse holds as well, if on proceeding to the excited state the polarity of the fluorophore should increase, then the hydrocarbon environment in which the probe is intercalated would be expected to inhibit the radiative lifetime of the equilibrium excited state.

Table 5. Representative Observed and Calculated Preexponential Coefficients for the Bimodal Fluorescence Decay of N-methylquinolinium in Gel-chromatographed Dihexadecylphosphate Vesicles at 25°C.

	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t Percent	χ^2
Observed[a]	9.37	34	3.45	66	1.30
Observed[a]	9.71	26	3.64	74	1.28
Average	--	30 ± 4	--	70 ± 4	--
Calculated[b]	--	35	--	65	--

aIn 0.0100 M NaH_2PO_4 at pH = 7.45. The conditions for the gel-chromatographic filtration are described in Table 2.

bCalculated by assuming that the anionic DHP monomers ion-pair predominantly with the hydrophobic N-methylquinolinium which is adsorbed longitudinally and transversally with respect to the plane of the endovesicular interface: the effective areas occupied by the anionic headgroup and by the N-methylquinolinium are, respectively, 70 and 200 angstroms squared, as discussed in the text.

It is not difficult to demonstrate that upon populating the lowest excited singlet state, the polarity of N-methylquinolinium increases. For example, according to the data presented in Table 3, the fluorescence lifetime of N-methylquinolinium in the pure phosphate buffer, with an ionic strength about 0.0965, is 8.88 nanoseconds; upon addition of the strong, inert, electrolyte sodium perchlorate--up to a concentration about 0.0500 M--into the buffered solution, the ionic strength becomes 0.146 and the fluorescence lifetime rises to 10.6 nanoseconds. Clearly, the longer fluorescence time constant indicates that the excited state is stabilized in media of higher ionic force to suggest, in turn, that the polarity of the N-methylquinolinium increases in the equilibrium excited state. Therefore, when intercalated, as in transversal adsorption, the fluorescence lifetime of N-methylquinolinium is reduced from approximately 8.90 nanoseconds, in the pure phosphate buffer, to about 4.0 ± 0.5 nanoseconds among the nonpolar hydrocarbon chains.

On the other hand, the emission time constant for the longitudinal adsorption of the probes, from the measurement of many fluorescence decays, is 9.5 ± .3 nanoseconds. As suggested earlier, the longitudinal microenvironment requires considerably more contact with the internal aqueous pocket based, for instance, on the comparable 10.1 nanoseconds lifetime of the N-methylquinolinium bound to sodium dodecylsulfate micelles, whose single microenvironment for a hydrophobic probe consists of water-hydrocarbon interfaces. Moreover, the longitudinal lifetime, τ_L, at 9.5 ± .3 nanoseconds, is slightly longer than the fluorescence time-constant in the pure phosphate

buffer, at 8.90 nanoseconds. This observation likely indicates that, as in the case of the intravesicular quencher toluenesulfonate, both mono- and dihydrogen phosphate are partly excluded from the intravesicular space, as a result of unfavorable electrostatic repulsions with the anionic surfaces of incipient vesicles. The partial exclusion of $H_2PO_4^{-1}$ and HPO_4^{-2} from the intravesicular space will lengthen the lifetime of the longitudinal adsorbates, relative to the fluorescence time constant in the pure phosphate buffer, because as the data in Table 3 demonstrate, the presence of the phosphate anions in 0.0100 M NaH_2PO_4 at pH = 7.45 deactivates the lifetime of N-methylquinolinium. For example, in pure water τ_f is 14.4 nanoseconds while upon addition of the phosphate buffer, τ_f is reduced to 8.88 nanoseconds.

By contrast, with the vesicles in their final assembled state, the longitudinal adsorbates are accessible to anionic deactivators such as toluenesulfonate. This occurs because of the low negative charge density of the endovesicular surface, as a result of strong ion-pair formation between the hydrophobic N-methylquinolinium and the counterionic dihexadecylphosphate amphipaths anchored along the water-hydrocarbon reef.

It is noteworthy that the existence of longitudinal and transversal binding sites, as parts of the topography of dihexadecylphosphate vesicles is well established. As an example, Almgren[24] observed a bimodal fluorescence decay upon adsorption of tris(2,2'-bipyridine)ruthenium(II) on DHP vesicles, in their acidic form, which

he attributed to the presence of two distinct solubilization sites: an ionic binding domain where the fluorophore is readily accessible to hydrophilic deactivators, and the more hydrophobic sites, which include some contact with the hydrocarbon chains, and where the luminophores are well protected against deactivation with ionic quenchers.

Likewise, Whitten[41] and his searchers have observed the existence of interfacial and inclusion complexes using hydrophobic stilbene derivatives in the presence of phosphatidylcholine, or lecithin, and dihexadecylphosphate vesicles.

Finally, and most recently, Hurst[49] and his collaborators have obtained evidence of the presence of lateral and intercalated residence sites for hydrophobic derivatives of methylviologen in the presence of dihexadecylphosphate vesicles, at pH = 8.0.

Exovesicular Adsorption.--The measurement of fluorescence in the continuous emission mode provides a more sensitive tool than the temporal decay method for the detection of a small concentration of adsorbed fluorophores whose deactivation generates only a small change in the total emission of the suspension. This fact is demonstrated below. Upon addition of intervesicular thallium(I) neither the component time constants, τ_ℓ and τ_t, nor the corresponding probabilities, A_ℓ and A_t, in the decay equation experience a change outside experimental error. Consequently, in agreement with the analysis based on the addition of intervesicular quenchers such as bromide, the fluorescent emission arises predominantly from the intravesicular N-methylquinolinium.

Table 6. The Emission Lifetime of N-methylquinolinium in Gel-chromatographed[a] Dihexadecylphosphate Vesicles and in the Presence of Thallium(I) at 25°C.

Deactivator added	Region	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^{2b}
--	--	9.67	24	3.79	76	1.13
0.0276 M $T\ell NO_3$[c]	Intervesicular	8.79	20	3.55	80	1.16

[a]In 0.0100 M NaH_2PO_4 at pH = 7.45: 1.50 mL of the gel-chromatographed eluate is collected to minimize the presence of the intervesicular N-methylquinolinium found in the latter portion (0.50 mL) of the vesicle band.

[b]The lamp calibration curve, or instrument response function, is displaced two channels to the left of the sample decay, to minimize the value of the chi-squared, as explained in Table 4.

[c]The fluorescent emission of N-methylquinolinium is indifferent with respect to the presence of nitrate.

Nevertheless, a small decrease in the emission intensity is detected upon addition of intervesicular quenchers such as thallium(I) or bromide by observing the fluorescence using a continuous emission spectrophotofluorometer. By following the change in the emission intensity, at 25°C, as a function of added Tl^{+1} or Br^{-1} deactivators in the range between 3 and 30 millimolar, the Stern-Volmer graphs in Figures 9 and 10 are generated.

The Stern-Volmer quenching constants for the thallium and bromide deactivators in the pure phosphate buffer at pH = 7.45, from Figure 9, are 15.7 and 135 M^{-1}, respectively. Now, in comparison, from Figure 10, the apparent Stern-Volmer constants for the Tl^{+1} and Br^{-1} intervesicular deactivators are equal to 8.00 and 1.21 M^{-1}, respectively. Consequently, for the case of bromide, the 100-fold decrease in the quenching efficiency with respect to the homogeneous medium, indicates that some N-methylquinolinium is associated with an environment that likely corresponds to the anionic exovesicular surface where the comparatively high charge density tends to exclude the anionic deactivator. Conversely, the small concentration of N-methylquinolinium is much more accessible to the cationic thallium(I) quencher, as evidenced by the higher Stern-Volmer constant, K_{SV}, in respect to the bromide deactivator, to demonstrate that the fluorophore is adsorbed on the anionic exovesicular surface. Moreover, it is noteworthy that the presence of a strong electric field about the exovesicular surface is expected because the hydrophilic, predominant, sodium ions dissociate readily from the counterionic dihexadecylphosphate amphipaths anchored on the exovesicular interface.

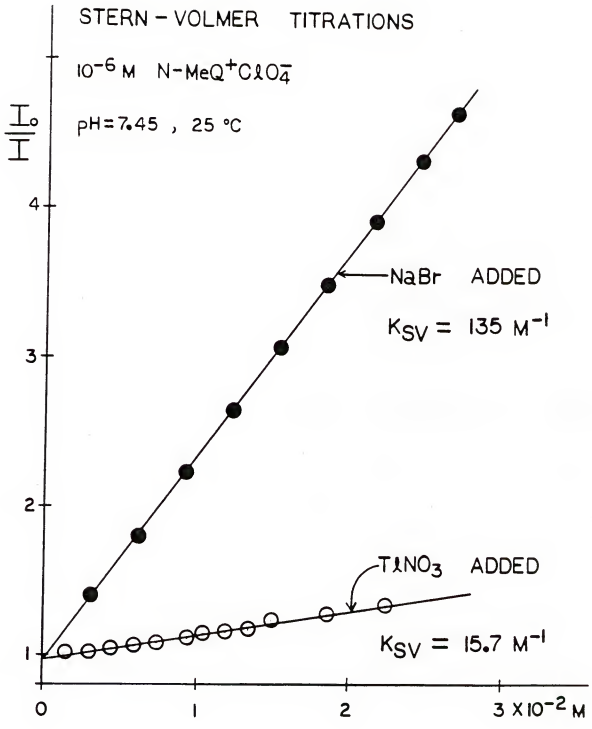


Figure 9. Stern-Volmer titrations of 1 μM N-methylquinolinium using $TlNO_3$ and NaBr.

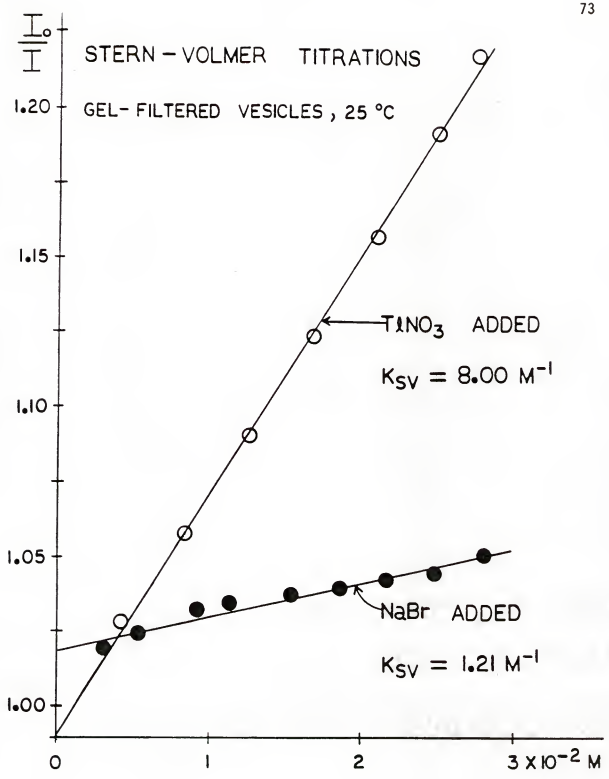


Figure 10. Stern-Volmer titrations of exovesicular N-methylquinolinium using $T\ell NO_3$ and NaBr.

By comparison, Almgren[24] has utilized the hydrophilic methylviologen dication MV^{+2}, at 25°C, to deactivate the fluorescence of tris(2,2'-bipyridine)ruthenium(II) adsorbed on the exovesicular surface of dihexadecylphosphate vesicles. Conversely, he has utilized intervesicular iodide, at 25°C, to inhibit the emission of pyrenebutyric acid adsorbed on the exovesicular surface of cationic didodecyltrimethylammonium bromide assemblies. In both cases, the observed decrease in the fluorescence is not the result of penetration of the assemblies by the deactivators, because from stopped-flow spectrophotometric measurements, Almgren showed that the hydrophilic quenchers methylviologen and iodide, do not penetrate the vesicles during the time required to complete the corresponding Stern-Volmer titrations. In addition, the impermeability of liposomes below their phase transition temperature to charged particles, or electrolytes, is expected.[50] For example, at neutral pH, below 35°C, dihexadecylphosphate vesicles are quite impermeable to electrolytes.[51]

Finally, as the work ahead shows, the presence of exovesicular solubilization sites is extremely important to understand the dynamics of transmembrane transport of N-methylquinolinium across dihexadecylphosphate vesicles at 45°C and pH = 7.45.

Thermal Redistribution Method

The above inventory of the distribution of the intravesicular fluorescent probes is based, in part, upon the relative surface areas of N-methylquinolinium and the anionic headgroup of dihexadecylphosphate, to provide an estimate of the mole fractions of the bimodal endovesicular states which corresponds with the magnitude of the

normalized coefficients in the biexponential fluorescence decay function. The correspondence strongly suggests the physical reality of the preexponential constants, in respect to the probability, or relative concentration, of the component lifetimes in the distribution.

As mentioned in the Introduction, to further demonstrate the relationship between the magnitude of the statistical weight coefficients and the probability of transversal and lateral adsorption, it is useful to induce a reversible redistribution in the frequency of the component lifetimes, to correlate the resulting change in the fluorescent emission with the variation in the magnitude of the normalized frequency factors A_ℓ and A_t.

Perhaps the most effective means to redistribute the component fluorescence lifetimes requires the thermal incubation of the vesicle suspension for increasing periods of time. An increase in the temperature facilitates the extrusion of N-methylquinolinium along its large, outwardly directed, concentration gradient. For instance, dihexadecylphosphate vesicles, at neutral pH, are known to become permeable to charged particles such as tris(2,2'-bipyridine)ruthenium(II) and the hydrophilic methylviologen dication, starting about 35°C, which probably represents the onset of the thermotropic phase transition, at neutral pH.[51] Accordingly, in the present work, to facilitate the extrusion of N-methylquinolinium, the dihexadecylphosphate vesicles are incubated at 45°C such that the hydrocarbon bilayer is likely to exist in the fluid state.

Now, the fluorescence lifetimes, τ_t, and τ_ℓ, as well as the corresponding frequency factors, A_t and A_ℓ, generated upon incubation

at 45°C, are recorded in Table 7. There are some important trends, or properties, in these data: the mole fraction, or contribution to the fluorescence decay, from the longer-lived component, T_ℓ, increases gradually with incubation time; note thereby, in the unheated suspension, about 30% of the emissive states correspond to the lateral endovesicular adsorbate, whose lifetime T_ℓ is 9.50 nanoseconds. After about 100 hours of incubation, the mole fraction of the longer-lived excited states has increased to about 70%.

In addition, the lifetime T_ℓ decreases monotonically to a value which is virtually identical to the lifetime, 8.88 nanoseconds, of the fluorophore in the pure phosphate buffer; note thereby, from the data in Table 7, that in the unheated suspension T_ℓ is 9.50 nanoseconds, while after 100 hours at 45°C, T_ℓ becomes 8.90 nanoseconds.

Finally, and most importantly, even after an incubation period of 100 hours, the fluorescence decay function remains bimodal. Thus, the increase in the probability of T_ℓ; the fact that this lifetime gradually becomes equal to the time constant of the fluorophore in the pure phosphate buffer; and the bimodal character of the decay function after prolonged incubation, may be taken as evidence that the fluorophore is equilibrated between the intravesicular and intervesicular volumes. But hardly so, because the intervesicular volume is exceedingly large, with respect to the intravesicular space. Therefore, the driving force, or concentration gradient, is extremely favorable towards the unidirectional release of N-methylquinolinium into the aqueous phase. To illustrate this effect, suppose that the suspension contains mostly large vesicles, about 1400 angstroms in diameter, then

Table 7. Thermal Redistribution of the Component Excited States of N-methylquinolinium in Gel-chromatographed[a] Dihexadecylphosphate Vesicles, Upon Incubation at 45°C.

Incubation time, hr[b]	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^2
0	9.54	30	3.54	70	1.29
20	9.38	51	3.73	49	1.12
40	9.33	58	3.87	42	1.15
60	9.30	57	3.94	43	0.983
80	9.12	70	4.30	30	0.985
100	8.91	73	3.89	27	1.14

[a]In 0.0100 M NaH_2PO_4 pH = 7.45. The conditions for the gel-chromatographic filtration are described in Table 2.

[b]After incubation at 45°C, the suspension is allowed to equilibrate to 25°C, for about 30 minutes, and then the fluorescence decay is measured.

from the total number of DHP monomers in each assembly, 1.6×10^5, and employing the concentration of phospholipid in the suspension, from Table 1, as 4.20×10^{-8} mole $\cdot$ cm^{-3}, the total volume, V_i, of the intravesicular space may be calculated

$$V_i = 4.20 \times 10^{-8} \text{ moles} \cdot \text{cm}^{-3} \times Vs \times N_A \times$$

$$\times \frac{1}{1.6 \times 10^5 \frac{\text{monomers}}{\text{vesicle}}} \times 1.15 \times 10^{-15} \frac{\text{cm}^3}{\text{vesicle}} =$$

$$= 4.0 \times 10^{-4} \text{ cm}^3.$$

Where Vs represents the volume of the gel-chromatographed suspension, 2.00 mL, and N_A stands for Avogadro's number.

Since the volume of the aqueous phase is 2.00 mL, it follows that when N-methylquinolinium diffuses out into the extravesicular space, about a 5000-fold dilution in its concentration takes place. Alternatively, if the suspension contains mostly 400 angstrom vesicles, the dilution following transmembrane extrusion is a staggering 30,000-fold. Consequently, because of the extreme dilution in the concentration of the fluorophore, as it diffuses into the bulk aqueous space, the discharge of N-methylquinolinium into the extravesicular medium ought to proceed to completion, which would invariably result in a single exponential decay with a relaxation constant equal to 8.88 nanoseconds, that is, the fluorescence lifetime of the free N-methylquinolinium in the pure phosphate buffer at pH = 7.45.

Nevertheless, consider the facts, only in more detail, from the data in Table 7. The fluorescence intensity is observed to increase rapidly during the first 20 hours of incubation at 45°C; note thereby, the increase in the mole fraction of T_ℓ from 30% at the beginning of the incubation to about 50% after incubation for 20 hours, at 45°C. Further incubation of the suspension for an additional 20 hours increases the mole fraction of T_ℓ only by a small amount, up to about 60%. Beyond 40 hours of incubation at 45°C, the change in the frequency of T_ℓ is very small, increasing to about 70% after 100 hours at 45°C. Clearly, then, the fact that the fluorescence decay remains bimodal, even after prolonged heating at 45°C, indicates that the transmembrane permeation does not proceed to completion which, in turn, suggests that the fluorescent probes are not being discharged into the main intervesicular volume.

Alternatively, the foregoing conclusion can be reached by using a more laborious approach based on the magnitude of the transmembrane potential, at equilibrium, to calculate the partition constant for discharge of the heterocyclic cations into the bulk aqueous phase. Since at equilibrium, the fluorophore is distributed between the intra- and intervesicular volumes--as the hydrophobic, readily permeating, cations diffuse out into the main aqueous volume as fast as they diffuse back into the organized assemblies--there is associated with this partition some transmembrane potential difference. Employing an equation first derived by Nernst in 1888, it is not difficult to appreciate that the magnitude of the transmembrane potential difference associated with the equilibrium distribution of

N-methylquinolinium is approximately given by the logarithmic quotient between the intra- and intervesicular volumes. Thus, if the suspension contains mostly 1400 angstrom assemblies, the transmembrane potential becomes

$$\Delta U = \frac{RT}{F} \ln\left(\frac{4.0 \times 10^{-4}\ cm^3}{2.00\ cm^3}\right) = -2.3 \times 10^2\ mV$$

where the constant RT/F amounts to 27 millivolts at 45°C. In comparison, when the suspension contains mostly 400 angstrom vesicles, the transmembrane potential is about -2.8×10^2 mV.

Still further, the negative sign associated with the preceding potentials denotes that the intravesicular space is not electrically balanced, or neutral, as it carries a microscopic excess of negative charge because of the tendency of N-methylquinolinium to diffuse along the large, outwardly directed, concentration gradient. Hence, because the interior of the vesicles is negatively charged, the cationic fluorophore is more stable within the DHP assemblies, and the amount of such stabilization is given, certainly, by the magnitude of the transmembrane potential.

Accordingly, in the case of the 1400 angstrom organizates, the stabilization of N-methylquinolinium within the intravesicular compartment can be calculated directly, from the magnitude of the transmembrane potential at equilibrium,

$$\Delta G = -2.3 \times 10^{-1} \frac{\text{joules}}{\text{coulomb}} \times 96487 \frac{\text{coulombs}}{\text{mole}} \times \frac{1\ \text{kilojoule}}{1000\ \text{joules}} = -22\ \text{kj} \cdot \text{mole}^{-1}$$

This calculation suggests that the intravesicular heterocyclic cations are about 22 kj · mole^{-1} lower in energy than their intervesicular counterparts. By inversion, then, the free intervesicular N-methylquinolinium lies approximately 22 kj · mole^{-1} higher in energy than its intravesicular form.

Now then, at long last, the thermodynamic partition constant for the extrusion of N-methylquinolinium can be calculated, as written,

$$Q_i^+ \underset{\longleftarrow}{\xrightarrow{K_X}} Q_o^+ \qquad (11a)$$

in which Q_i^+ represents the fluorophore within the vesicles and Q_o^+ symbolizes the free, intervesicular, N-methylquinolinium.

Since the latter state lies about 22 kj · mole^{-1} higher in energy than the intravesicular one, the partition constant for extrusion into the aqueous phase is given by

$$K_X = \frac{[Q_o^+]}{[Q_i^+]} = e^{\left(\frac{-2.2 \times 10^4 \text{ joule} \cdot \text{mole}^{-1}}{8.31 \text{ joule} \cdot \text{mole}^{-1} \, ^\circ K^{-1}} \cdot \frac{1}{318 \, ^\circ K}\right)} =$$

$$= 2.4 \times 10^{-4}.$$

The small value of K_X proves, therefore, that the extent of transmembrane diffusion into the bulk aqueous phase at 45°C is very small. Consequently, the considerable increase in the fluorescence intensity, following incubation at 45°C, should not result from an equilibrium distribution of the luminescent cations between the intravesicular and the main aqueous phases.

Alternatively, the above observation concerning the presence of a small fraction of the fluorophore adsorbed on the outer surface of the vesicles suggests that the N-methylquinolinium may be distributing itself across the hydrocarbon bilayer, between the endo- and exovesicular surfaces, according to the process

$$Q_e^+ \underset{}{\overset{K}{\rightleftharpoons}} Q_x^+ \qquad (11b)$$

in which Q_e^+ represents the endovesicular fluorophore and Q_x^+ its exovesicular state; K represents the corresponding partition constant for the distribution of the probe between the inner and outer surfaces.

The previous observation regarding the bimodal character of the fluorescence decay, even after continued incubation at 45°C, is now easily explained in terms of the two-state model presented in equation (11b). Accordingly, the outwardly directed permeation of N-methylquinolinium does not proceed to completion because the binding sites on the outer surface gradually become occupied, or saturated, with the fluorophores. Hence, the heterocyclic cations can jump, or permeate, across the hydrocarbon bilayer only when free sites are available on the exovesicular surface.

The phenomenon of surface saturation and blocking of transmembrane transport is also well documented in the case of hydrophobic ions. Consider, as an example, the work of Lauger[52] and his associates. They investigated the transmembrane partition of hydrophobic ions such as tetraphenylboron and the monoanion of dipicrylamine, across hydrocarbon bilayers, or black lipid membranes, made up of

dioleoylphosphatidylcholine--a phospholipid whose skeleton contains 18 carbon atoms, and a single double bond--in the presence of an imposed potential difference. On applying a step in the voltage, they observed an exponentially decaying current which they attributed to the redistribution of the hydrophobic anions across the bimolecular membrane according to the voltage gradient. Moreover, by measuring the conductance of the hydrocarbon bilayer as a function of the concentration of added permeant anions, Lauger[52] and his coworkers observed that the initially linear graph passed through a maximum at the higher concentrations of added tetraphenylboron or dipicrylamine monoanions. They interpreted this phenomenon readily in terms of the limited number of binding sites available on the surface of the membrane; or equivalently, in terms of the saturation of the surface according to the theory of transmembrane transport and blocking phenomena first proposed by Bruner. [53]

Furthermore, Lauger[52] and his associates showed that the adsorption of tetraphenylboron and the dipicrylamine anions is noncoulombic and, therefore, hydrophobic in its origin; finally, Lauger suggested that the binding of the hydrophobic anions probably takes place along the polar layer on the surface of the membrane where the headgroups are anchored.

Concerning the solubilization sites of N-methylquinolinium on the exovesicular surface of the dihexadecylphosphate assemblies, the gradually increasing mole fraction, A_{ℓ}, of the longer-lived time constant, and the fact that the fluorescence decay remains bimodal during incubation, as the probes become distributed between the

inner and outer surfaces, suggests strongly that the time constant of the exovesicular excited states is very nearly degenerate with the longitudinal, endovesicular fluorescence lifetime. Consequently, the exovesicular fluorophore must be adsorbed longitudinally with respect to the plane of the outer surface of the DHP assemblies.

Conversely, transversal or intercalated adsorption on the outer surface of the vesicles is absent because upon gel-chromatographic filtration, there is left a large excess of sodium cations in the intervesicular space such that the exovesicular anionic headgroups ion-pair preferentially with sodium ions; thus to prevent significant intercalation of N-methylquinolinium among the hydrocarbon chains of the surfactant monomers on the external surface of the vesicles.

The foregoing analysis demonstrates the presence of an inherent asymmetry between the outer and inner surfaces of the DHP vesicles. It is significant that Furlong[54] and his collaborators have observed a very similar transmembrane asymmetry in reference to the adsorption of tris(2,2'-bipyridine)ruthenium(II) on the inner and outer surfaces of dihexadecylphosphate assemblies. They observed, for instance, that the ruthenium complex adsorbed on the exovesicular surface has a fluorescence lifetime which is the same as its relaxation constant in aqueous solution, and suggested that the fluorophore experiences a binding environment which is free of steric constraints, whose polarity is alcohol-like, and, in general, very much like that found on the surface of a micelle.

By comparison, they observed that the fluorescence lifetime of the endovesicular sensitizer is longer than its relaxation constant in aqueous solution. Likewise, they reported that the amount of occluded sensitizer is greater than can otherwise be accommodated by the area of the inner surface alone; thus implying the presence of an alternative mode of adsorption along the inner vesicular ridge.

Finally, and importantly, they observed that the endovesicular emission of $Ru(bpy)_2^{+2}$ is displaced hypsochromically, relative to the position of the fluorescence in aqueous solution; suggesting that the endovesicular adsorbates are more constrained, with respect to their spatial freedom, than the exovesicular ones. They derived the latter conclusion from the similar, albeit larger, hypsochromic shift in the emission of $Ru(bpy)_2^{+2}$ adsorbed in the clay matrix of montmorillonite and which DellaGuardia and Thomas[55] have attributed to a configurational deformation in the geometry of the bipyridine ligands, as a result of intercalation of the sensitizer into the colloidal surface.

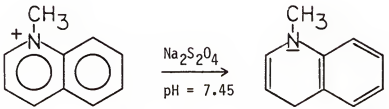
Certainly, the observations of Furlong and his associates[54] are quite consistent with the work described here. For example, their outer vesicular adsorbates, which experience an environment similar to that observed on the surface of a micelle, correspond adequately with the exovesicular adsorbates of N-methylquinolinium whose relaxation constant, as in the case of $Ru(bpy)_2^{+2}$, is the same as that observed in the bulk aqueous phase. Conversely, their more constrained, inner vesicular, states are equivalent to the transversal, or intercalated, endovesicular adsorbates of N-methylquinolinium.

However, it is noteworthy that Furlong[54] considers the asymmetric bilayer of DHP to be a consequence of a difference in the mode of packing of the amphipaths along inner and outer vesicular walls. Or, alternatively, as a result of a difference between the ionic strength within the inter- and intravesicular compartments.

Continuing further, the small but nevertheless monotonic decrease in the lifetime, τ_ℓ, from about 9.50 nanoseconds in the unheated suspension, to about 8.90 nanoseconds after 100 hours of incubation, is easily explained in terms of saturation of the exovesicular surface by the heterocyclic cations. Clearly, as the outer surface becomes saturated, there takes place a gradual decrease in the degree of dissociation of the anionic DHP monomers, and consequently, in the charge density of the external surface of the vesicles. Hence, the exovesicular surface does not inhibit the approach of the deactivating anions, $H_2PO_4^{-1}$ and HPO_4^{-2} which are present in the main aqueous phase, such that the lifetime of the cationic exovesicular adsorbates gradually becomes identical to the time constant of the fluorophores in the pure phosphate buffer at pH = 7.45.

Dithionite Reduction of the Exovesicular Adsorbates.--A useful method to continue to investigate the relationship between the magnitude of the preexponential coefficients A_ℓ and A_t in the bimodal decay function, and the respective mole fraction of the component excited states, requires the selective reduction of the exovesicular N-methylquinolinium by using sodium dithionite. This stratagem transforms the outer adsorbates into the nonfluorescent compound

1-methyl-4-dihydroquinoline according to the reaction



As an example of the stratagem, Hurst and his researchers[49] have demonstrated that dithionite is an effective agent for the reduction of hydrophobic derivatives of methylviologen adsorbed on the outer, or exovesicular, surface of dihexadecylphosphate vesicles at pH = 8.0. Moreover, because the strongly hydrophilic dithionite does not penetrate the hydrophobic bilayer of DHP vesicles, Hurst observed that the intravesicular methylviologen dications are protected against chemical reduction.

The effect of dithionite on the lifetime distribution of N-methylquinolinium in the presence of dihexadecylphosphate vesicles is described by the data in Table 8. For example, in the unheated suspension the longitudinal τ_ℓ and transversal τ_t lifetimes are 9.54 and 3.54 nanoseconds, respectively; while the corresponding longitudinal A_ℓ and transversal A_t normalized preexponential coefficients are, in turn, 30 and 70%. Furthermore, upon prolonged incubation at 45°C, the frequency of lateral adsorption, A_ℓ, gradually increases to approximately 60%, as the fluorophores redistribute between the internal and external surfaces of the organized assemblies. By contrast, when sodium dithionite is introduced into the incubated

Table 8. Dithionite Reduction of Exovesicular N-methylquinolinium, at pH = 7.45, after Prolonged Incubation at 45°C.

Incubation time, hr[a]	$[S_2O_4^=]$, M[b]	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^2
0	--	9.54	30	3.54	70	1.29
40	--	9.16	60	3.64	40	1.06
40	6.0×10^{-5}	9.76	31	3.62	69	1.33

[a]The fluorescence decays are measured at 25°C.

[b]Anaerobic conditions: upon addition of the sodium dithionite, the suspension is reincubated for 2 hours at 45°C. After equilibration to 25°C, the absorbance at 315 nm is found to be 0.180; thus, taking the extinction coefficient of dithionite about 6900,[49] the remaining concentration of $S_2O_4^=$ is approximately 2.60×10^{-5} M.

suspension, the fluorescence intensity decreases considerably. In addition, the frequency distribution reverts in time such that the normalized coefficients become identical to those observed in the unheated suspension. Thus, Table 8 indicates that upon introducing $S_2O_4^=$ into the incubated, anaerobic, suspension A_ℓ and A_t return to 30 and 70%, respectively.

On account of the fact that dithionite is unable to penetrate the DHP assemblies, it is clear that upon reduction of the exovesicular adsorbates, only the intravesicular fluorophores remain to be observed. Therefore, the fact that the relative probabilities A_ℓ and A_t of the endovesicular fluorophores remain constant, even after prolonged incubation, indicates that the longitudinal and transversal adsorbates exist in a state of dynamic equilibrium.

Moreover, the state of chemical equilibrium proves that the bimodal fluorescence decay is not related to the binary distribution in the diameter of the vesicles observed by dynamic turbidimetry, since it is otherwise cumbersome to invoke a dynamic equilibrium between excited states which are secluded in separate vesicles, particularly in a dilute suspension.

Nevertheless, the existence of a dynamic equilibrium between the intravesicular component adsorbates is of fundamental importance, because it introduces the simplifying element from which the extent of permeation, or transmembrane transport, at equilibrium may be predicted for a vesicle of any diameter. Accordingly, from a knowledge of the equilibrium distribution of the intravesicular excited states including the surface area occupied by N-methylquinolinium and the

total number of heterocyclic cations associated with the organizate, it is easy to predict the distribution of the fluorophores at equilibrium for a vesicle of any size; thus to introduce the necessary quantitative spine into the concept of the transmembrane distribution of N-methylquinolinium between the endo- and exovesicular surfaces.

Consider, therefore, the 400 angstrom organizates. The number of DHP monomers along the endovesicular surface is calculated about 4.0×10^3, by taking the length of the hydrophobic bilayer as 50 angstroms, and using the 70 angstroms squared suggested by Hurst[45] as the surface area of the anionic DHP monomers. Furthermore, as indicated above, the anionic amphipaths ion-pair predominantly with the N-methylquinolinium because, while the vesicles are prepared, both the N-methylquinolinium and the alternative sodium counterions are present in comparable concentrations such that the anionic water-hydrocarbon interfaces select the more hydrophobic species, namely, the heterocyclic cations. Consequently, by electrical neutrality, the initial number, N_e^o, of endovesicular fluorescent probes in the unheated, gel-chromatographed, assemblies is also 4.0×10^3.

Now, recalling the model in question, upon prolonged incubation at 45°C, the heterocyclic cations will slowly distribute themselves between the endo- and exovesicular surfaces until the outer interface is coated with a monolayer of the probes; thus the transmembrane diffusion of the fluorophore eventually ceases since the initially free exovesicular binding sites become occupied with the fluorescent adsorbates. Accordingly, at saturation, the exovesicular fluorophore is adsorbed longitudinally with respect to the plane of the interface;

as indicated from its comparatively long, 8.90 nanoseconds, time constant which is nearly degenerate with the longitudinal, endovesicular, 9.54 nanoseconds lifetime, such that the fluorescence decay remains bimodal throughout the incubation period.

It, therefore, follows that the maximum number, N_{ℓ}^{x}, of N-methylquinolinium ions adsorbed on the exovesicular surface is readily calculated from a knowledge of the surface area per adsorbed N-methylquinolinium--estimated as 200 angstroms squared, in analogy with the area of the geometrically equivalent methylviologen dication--and the external area, A_x, of the assemblies,

$$N_{\ell}^{x} = \frac{Ax}{2.0 \times 10^{2}\ \mathring{A}^{2}} = \frac{4\pi(200\ \mathring{A})^{2}}{2.0 \times 10^{2}\ \mathring{A}^{2}} = 2.5 \times 10^{3}.$$

Hence, after prolonged incubation at 45°C, the number of endovesicular heterocyclic cations, Ne, becomes

$$Ne = N\mathring{e} - N_{\ell}^{x} = 4.0 \times 10^{3} - 2.5 \times 10^{3} = 1.5 \times 10^{3}$$

Furthermore, since the dithionite reduction stratagem indicates that the mole fractions of the component internal adsorbates are constant, then the number of longitudinal, N_{ℓ}^{e}, and transversal, N_{t}^{e}, endovesicular adsorbates is

$$N_{\ell}^{e} = 0.30 \times Ne = 4.5 \times 10^{2} \text{ and}$$

$$N_{t}^{e} = 0.70 \times Ne = 1.1 \times 10^{3}.$$

At last, recalling that the lifetimes of the longitudinal endovesicular and exovesicular adsorbates are nearly degenerate, the mole fraction, A_t, of the longer-lived, laterally adsorbed

N-methylquinolinium, at saturation, is

$$A_{\ell} = \frac{N_{\ell}^{x} + N_{\ell}^{e}}{N_e^{\circ}} = \frac{2.5 \times 10^3 + 4.5 \times 10^2}{4.0 \times 10^3} = 0.74$$

and therefore the corresponding mole fraction, A_t, of the shorter-lived intercalated N-methylquinolinium, after continued incubation at 45°C, is 0.26.

Consider, similarly, the 1400 angstrom organizates. The number of DHP monomers along the endovesicular surface is about 7.6×10^4. Again, the principle of electrical neutrality requires the same number, N_e°, of fluorescent N-methylquinolinium probes to be adsorbed initially along the internal hydrophobic-hydrophilic interface.

Likewise, at saturation, the maximum number, N_{ℓ}^{x}, of fluorescent probes which are adsorbed longitudinally on the exovesicular surface is readily calculated,

$$N_{\ell}^{x} = \frac{A_x}{2.0 \times 10^2 \text{ Å}^2} = \frac{4\pi(7.0 \times 10^2 \text{ Å})^2}{2.0 \times 10^2 \text{ Å}^2} = 3.1 \times 10^4$$

Hence, after prolonged incubation, the number, N_e, of endovesicular heterocyclic cations becomes

$$N_e = N_e^{\circ} - N_{\ell}^{x} = 7.6 \times 10^4 - 3.1 \times 10^4 = 4.5 \times 10^4.$$

Then, according to the equilibrium distribution of the internal component excited states determined from the dithionite reduction method, the number of longitudinal, N_{ℓ}^{e}, and transversal, N_t^{e}, endovesicular adsorbates are

$N_{\ell}^{e} = 0.30 \times N_e = 1.3 \times 10^4$ and

$N_t^e = 0.70 \times N_e = 3.1 \times 10^4$

Finally, at equilibrium, the mole fraction, A_{ℓ}, of the longitudinally adsorbed N-methylquinolinium is

$$A_{\ell} = \frac{N_{\ell}^{x} + N_{\ell}^{e}}{N_e^{o}} = \frac{3.1 \times 10^4 + 1.3 \times 10^4}{7.6 \times 10^4} = 0.58$$

therefore the corresponding mole fraction, A_t, of the shorter-lived intercalated N-methylquinolinium, after continued incubation at 45°C, is 0.42.

Table 9 lists the mole fractions observed by experiment upon prolonged heating of the suspension at 45°C. The respective values of A_{ℓ} and A_t represent the average mole fractions of the longitudinal and transversal adsorbates, as calculated from the data in Table 7, for the incubation range between 40 and 100 hours. Thus the average longitudinal mole fraction, $\bar{A}_{\ell}$, is 64% while for transversal adsorption, $\bar{A}_t$ is equal to 36%.

For comparison, Table 9 also contains the respective mole fractions of the longitudinal and transversal adsorbates, as calculated from the preceding inventory based on the equilibrium between the endo- and exovesicular surfaces. Although the relative concentrations of the large, 1400 angstroms, and small 400 angstroms, vesicles have not been determined, it is useful to suppose that both assemblies are present in equimolar concentrations in the suspension, such that they contribute equally to the average extent of transmembrane permeation,

Table 9. Observed and Calculated Preexponential Coefficients for the Bimodal Fluorescence Decay of N-methylquinolinium in Gel-filtered DHP Vesicles after Prolonged Incubation at 45°C.

	Incubation time, hr	Vesicle Diameter in Angstroms	τ_ℓ, ns	$\bar{A}_\ell$, Percent	τ_t, ns	$\bar{A}_t$, Percent	χ^2
Observed[a]	40 - 100	--	9.16 ± .15	64 ± 7	4.00 ± .15	36 ± 7	<1.15
Calculated[b]	--	400	--	74	--	26	--
Calculated[b]	--	1400	--	58	--	42	--
Average	--	--	--	66 ± 8	--	34 ± 8	--

[a]The mean values and uncertainties of the lifetimes and the preexponential coefficients are obtained from the data in Table 7.

[b]Based upon the transmembrane equilibration of N-methylquinolinium between the endo- and exovesicular surfaces: the areas occupied by the anionic headgroup and the fluorophore are 70 and 200 angstroms squared, respectively, as discussed in the text.

at equilibrium. When calculated in this manner, the mean equilibrium value for longitudinal adsorption, A_{ℓ}, is equal to 66% while in the case of transversal adsorption, A_t, is equal to 34%.

The agreement between the frequencies of the component excited states thus calculated and the values observed by experiment serves to illustrate the accuracy of the concept regarding the transmembrane distribution of the fluorescent probes between the inner and outer surfaces of the dihexadecylphosphate assemblies. Accordingly, a schematic description of the transmembrane equilibration is given in Figure 11.

Thermal Deactivation Method

Although the intrinsic rate constant of fluorescence, k_f^o, and therefore the corresponding inherent radiative lifetime, τ_o, are independent of temperature, the measured or apparent lifetime, τ_{app}, can be a function of this parameter. As Turro[56] has suggested, the temperature dependence arises when the upper vibrational levels of the excited state possess some more efficient mechanism for radiationless relaxation than the lowest vibrational state. The route of such radiationless deactivation of the excited state manifold is generally associated with the existence of an activation barrier for intersystem crossing or, alternatively, the activation barrier might be associated with the rate of internal conversion. The presence of both of these radiationless paths is evident from the definition of the measured, or apparent, lifetime τ_{app}

$$\tau_{app}^{-1} = k_f^o + k_x + k_{ic} \qquad (12)$$

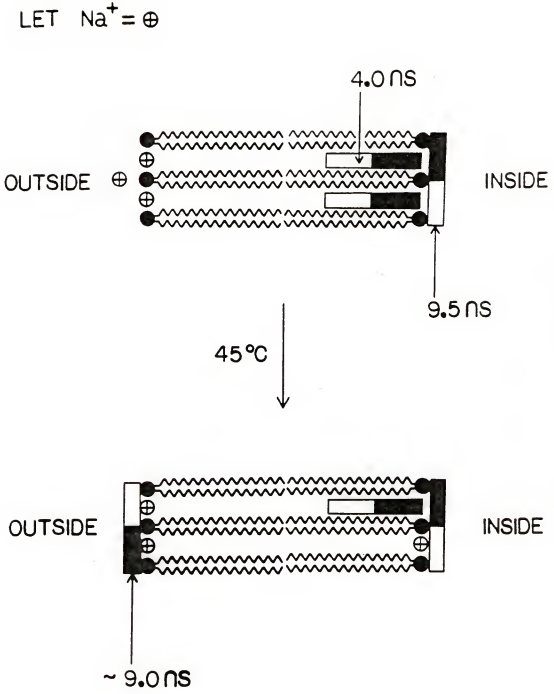


Figure 11. Schematic distribution of N-methylquinolinium in dihexadecylphosphate vesicles.

where k_f° signifies the unimolecular, natural, rate constant of emission while k_x and k_{ic} represent, respectively, the rate constants of intersystem crossing and internal conversion. Thus when an activation barrier is associated, for example, with the rate constant of intersystem crossing, k_x becomes dependent on the temperature while k_f° and k_{ic} remain fixed; therefore,

$$\tau_{app}^{-1} = \text{constant} + k_x. \tag{13}$$

Now, the Arrhenius equation for the thermal dependence of unimolecular rate constants is as follows,

$$k_x = Ae^{-\frac{Ea}{RT}} \tag{14}$$

where Ea represents the activation energy, associated in this case with intersystem crossing, and A is the corresponding preexponential factor. On combining equations (13) and (14), the result obtains

$$\tau_{app}^{-1} = \text{constant} + Ae^{-\frac{Ea}{RT}} \tag{15}$$

If the energy term in equation (15) is large, then the relationship is simplified to yield

$$\tau_{app}^{-1} = Ae^{-\frac{Ea}{RT}}$$

Hence, a plot of the natural logarithm of τ_{app}^{-1} versus the reciprocal absolute temperature will be linear, to provide the appropriate activation energy for intersystem crossing. In this regard, it is of interest that the apparent fluorescence time constant of

N-methylquinolinium is found to depend upon the temperature and to provide a linear Arrhenius plot in the range between 5° and 45°C. As suggested in the Introduction, such a temperature dependence provides a useful tool to continue to characterize the binding environments of the intravesicular probes.

Table 10 describes the effect of temperature on the fluorescence decay in the range between 5° and 45°C for a suspension of gel-chromatographed vesicles which has been previously incubated for 70 hours at 45°C. While the dynamic equilibrium between the two component lifetimes is not influenced by the temperature--as evidenced by the constant values of the component mole fractions--the respective emission time constants nevertheless increase as the temperature is reduced. Note accordingly, at 45°C, the mole fractions A_ℓ and A_t are, in turn, 77 and 23% while the corresponding fluorescence lifetimes T_ℓ and T_t are 6.72 and 2.26 nanoseconds, respectively. By comparison, at 5°C, the mole fractions A_ℓ and A_t remain constant at 72 and 28%, respectively, while the lifetimes T_ℓ and T_t increase, in turn, to 11.3 and 4.82 nanoseconds.

Furthermore, from the Arrhenius parameters, recorded similarly in Table 10, it is observed that the longitudinal lifetime T_ℓ has an activation energy about 2.30 kcal/mole and an entropy of activation equal to 11.8 cal/(mole °K); in contrast, the activation energy of the transversal, or intercalated lifetime, T_t, is 3.35 kcal/mole with an entropy of activation about 17.2 cal/(mole °K).

Table 10. The Influence of Temperature on the Fluorescence Lifetimes of N-methylquinolinium Adsorbed on Dihexadecylphosphate Vesicles[a] after Incubation for 70 Hours at 45°C.

T, °K	T_ℓ, ns	A_ℓ, Percent	T_t, ns	A_t, Percent	χ^2	Arrhenius Parameters: Ea, Kcal/mole T_ℓ[b]	Ea, Kcal/mole T_t[c]	$\Delta S^{\neq}$, cal/mole·°K T_ℓ	$\Delta S^{\neq}$, cal/mole·°K T_t
318	6.72	72	2.26	28	1.08	2.30	3.35	11.8	17.2
298	9.02	67	3.83	33	1.06				
298	8.85[d]	72	3.91	28	1.09				
278	11.3	77	4.82	23	0.870				

[a] In 0.0100 M NaH_2PO_4 at pH = 7.45. Gel-chromatographed according to the conditions described in Table 2.
[b] The coefficient of correlation is 0.997.
[c] The coefficient of correlation is 0.964.
[d] Incubated 105 hours at 45°C.

For the purpose of comparison, the Arrhenius parameters of the excited state of N-methylquinolinium in the pure phosphate buffer, at pH = 7.45, are listed in Table 11. The corresponding activation barrier is about 2.38 Kcal/mole with an activation entropy equal to 12.2 cal/(mole °K). These values agree with the Arrhenius parameters of the longer-lived relaxation constant in the bimodal decay to suggest, again, that the emissive states which generate ℓ experience considerable contact with an aqueous environment.

Osmotic Tension Method

It has been previously mentioned that organized assemblies such as liposomes are sensitive to a change in the osmolarity of the aqueous medium in which they are suspended. For example, when the organizates are suspended in a hyperosmolar medium whose salt concentration exceeds that of the solution in which the vesicles are originally prepared, the assemblies contract, hence the salt concentration within the internal pocket of water becomes identical to the concentration in the main volume of the aqueous phase. Conversely, when the organizates are suspended in a hypoosmolar medium whose salt concentration is lower than that of the medium in which the vesicles are assembled for the first time, the structures expand in order to maintain the same osmolarity, or salt concentration, across the semipermeable membrane.

Thus, if a large change in the size of the assemblies might be induced, on changing the ionic strength, it is likely that the respective lifetimes and mole fractions would be altered. Therefore, the influence of the ionic force on the fluorescence decay of the unheated,

Table 11. The Influence of Temperature on the Fluorescence Lifetime of N-methylquinolinium[a] in 0.0100 M NaH_2PO_4 at pH = 7.45.

			Arrhenius Parameters	
T, °K	A	τ, ns	Ea, Kcal/mole[b]	$\Delta S^{\neq}$, cal/(mole °K)
318	0.78	6.71	2.38	12.2
308	0.75	7.72		
298	0.96	8.88		
278	0.72	11.7		

[a]Micromolar solution.
[b]The coefficient of correlation is 0.999.

gel-chromatographed suspension of vesicles is recorded in Table 12. As an example, a threefold increase in the ionic strength of the suspension can be readily induced on addition of the strong, inert, electrolyte sodium perchlorate up to a concentration about 0.0500 M; whereupon the values of A_ℓ and A_t are observed to be 26 and 74%, with T_ℓ at 10.5 nanoseconds and T_t at 3.57 nanoseconds, respectively. On the other hand, when the ionic strength of the suspension is lowered threefold by diluting appropriately with degassed, distilled, water a set of parameters is obtained which is the same, within the experimental uncertainty, as when 0.0500 M sodium perchlorate is added to the suspension.

Consequently, it appears that a threefold increase or decrease in the ionic strength is not sufficiently strong to generate a large change in the physicochemical state of the gel-chromatographed, unheated, organizates. Yet, larger perturbations are not recommended because, for instance, an increasing dilution will eventually lyse, or rupture, the vesicles; conversely, an increasing ionic strength might precipitate them out of the suspension.

N-methylquinolinium as a Fluorometric Sensor of Membrane Potential

Consider now a suspension of pure, gel-chromatographed, dihexadecylphosphate vesicles in 0.0100 M NaH_2PO_4, at pH = 7.45 and at 45°C, whose total surfactant concentration is 4.20×10^{-5} M. Suppose furthermore that an aliquot of a stock solution of N-methylquinolinium is added into the suspension to give, for example, a micromolar intervesicular concentration in the fluorophore. The question may be

Table 12. The Influence of Osmolarity on the Fluorescence Lifetimes of N-methylquinolinium within Dihexadecylphosphate Vesicles[a] at 25°C.

	Ionic Strength	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^2[b]
Undiluted	0.0229	9.80	28	3.79	72	1.13
3-Fold diluted[c]	0.00763	11.0	30	3.76	70	1.21
0.0500 M $NaClO_4$[d]	0.0729	10.5	26	3.57	74	1.11

[a]In 0.0100 M NaH_2PO_4 at pH = 7.45. Gel-chromatographed according to the conditions described in Table 2.

[b]The lamp profile or instrument response function is displaced one channel to the left of the sample decay.

[c]An aliquot of the gel-chromatographed eluate is diluted 3-fold using degassed, distilled, water and the fluorescence decay is measured immediately thereafter.

[d]Sodium perchlorate is added into an aliquot of the gel-chromatographed suspension. The fluorescence decay is measured after equilibrating the suspension for 10 hours at 25°C.

brought up as to whether the heterocyclic cations will penetrate the DHP assemblies under these conditions. This is a central question because it helps to illustrate the most important, direct, practical application of the work presented here, namely, the use of N-methylquinolinium as a probe of membrane potential. Sensors of this type are invaluable, because they provide the fluorometric alternative to the penetration of cells and other organelles with cumbersome and potentially damaging microelectrodes.

Naturally, in the case at hand, the hydrophobic N-methylquinolinium will diffuse into the pure DHP vesicles if an inwardly directed concentration gradient is present. In this respect, the volume of the chromatographed suspension is 2.00 mL; as the concentration of intervesicular fluorophore is micromolar, there is about two nanomoles of N-methylquinolinium in solution. Now, recalling that in a suspension of 1400 angstrom assemblies, at 4.20×10^{-5} M dihexadecylphosphate, the intravesicular volume is estimated as 4.0×10^{-4} cm^3, then, when the cationic probes diffuse into the assemblies, the resulting concentration of intravesicular N-methylquinolinium will be about 5.0×10^{-3} M, which corresponds to a 5000-fold increase with respect to the micromolar intervesicular concentration. Furthermore, regarding the smaller 400 angstrom assemblies, diffusion of N-methylquinolinium from the bulk aqueous phase into the vesicles results in a 30,000-fold increase in the concentration of the probes. It follows, therefore, that the unfavorable concentration ramp, or gradient, precludes diffusion of the luminophore into the organized assemblies.

Nevertheless, there is a means, or rather a method, whereby the N-methylquinolinium can be induced to permeate into the liposomes, against the unfavorably steep concentration ramp. The method consists of polarizing the vesicles--inside negative--by creating a transmembrane electric potential with which to pump the heterocyclic cations inwardly; up against a concentration gradient, yet down along an electric potential difference. Since in this case N-methylquinolinium is acting as the voltage sensor, an inside negative transmembrane potential can only be generated by establishing an outwardly directed sodium ion diffusion potential. This, in turn, can be brought about by diluting the vesicle suspension at constant ionic strength, in some buffered medium lacking sodium ions. Under these conditions, and especially above the thermotropic phase transition, some sodium ions will diffuse outwardly, under the influence of their concentration slope, to impress a negative charge on the interior of the assemblies, thus polarizing them. Accordingly, the hydrophobic, readily permeating, fluorescent probes respond to the transmembrane potential gradient, or voltage, by diffusing into the vesicles, whereupon their fluorescence intensity is quenched because the lifetime of the predominant endovesicular emitters, 3.8 nanoseconds, is smaller than

the relaxation constant, 8.88 nanoseconds, of the free fluorophore in the pure phosphate buffer, at pH = 7.45.

Still, while the transmembrane diffusion of N-methylquinolinium is comparatively rapid, because of its hydrophobicity, the rate of outward permeation of sodium ions is, at best, sluggish, because it is quite a hydrophilic specie. Consequently, the change in fluorescence observed as the luminophores penetrate the assemblies is rather slow, since the rate at which the vesicles are polarized is determined by the slower escape of sodium ions. However, there is a means to increase the rate of membrane polarization which consists of incorporating a sodium carrier, or ionophore, into the hydrophobic bilayer, to assist in the transmembrane escape of Na^+, thus to polarize the vesicles much more rapidly and efficiently.

Of course, the use of luminescent cations as probes of membrane potential is not a new field, and the preceding discussion now represents common knowledge in the area of bioelectrochemistry. Consider, by way of an illustration, the work of Waggoner[57] who, in the early seventies, pioneered the introduction of fluorescent probes as membrane potential sensors.

Waggoner and his collaborators[57] have examined 29 symmetric cyanine dyes for possible use as fluorometric potential probes in the presence of phosphatidylcholine vesicles and similarly in

the presence of phosphatidylcholine vesicles and similarly in the presence of human red blood cells. In this regard, the most sensitive membrane voltage probe turns out to be the hydrophobic dye diS-C_3-(5). The nomenclature indicates that the symmetric cyanine dye contains a sulfur atom in the nucleus; furthermore, the nitrogen nucleus contains an alkyl chain of three carbon atoms; finally, the cationic probe incorporates five methine units. Now, the properties which make it possible for diS-C_3-(5) to serve as a membrane potential sensor include, of course, its hydrophobicity, whereby the structure can permeate freely across nonpolar bilayers; and the fact that its emission intensity becomes quenched upon penetration of the assemblies--as nonfluorescent aggregates are formed because, when constrained within the microscopic volume of the vesicles, the resulting local concentration of the dye is quite high.

Consider now a suspension of vesicles made up of 3:1 phosphatidylcholine-cholesterol, containing about 10^{-3} M phospholipid, prepared and suspended in the presence of potassium ions such that the intra- and intervesicular compartments are isotonic--that is, the concentration of potassium ions is the same inside as well as outside the vesicles. Waggoner and his associates[57] introduced subsequently diS-C_3-(5) into such a suspension, up to a micromolar concentration, whereupon the fluorescence intensity remained unchanged and the same as that in the absence of the liposomes. Naturally, since the

concentration of potassium ions is the same outside as well as inside, the vesicles remain electrically balanced, or neutral, and consequently the cyanine probe is altogether indifferent to their presence.

Waggoner[57] then proceeded to suspend the vesicles in an isotonic, potassium-free, medium to generate an outwardly directed diffusion potential, while changing neither the ionic strength nor the pH of the intervesicular environment. As the potassium ions now tend to diffuse along their concentration gradient, they impress a negative charge on the liposomes and the membrane becomes polarized--inside negative. As expected, the cationic cyanine dyes then respond to the transmembrane electric potential by diffusing into the vesicles, as suggested by the observed 30% reduction in the fluorescent emission.

The reduction in fluorescence, however, is sluggish; occurring over a period of 10 minutes, because the hydrophobic bilayer retards the passage of potassium ions. By contrast, when valinomycin, the macrocyclic depsipeptide made up of alternating alpha-aminoacids and alpha-hydroxyacids, that functions as a specific membranogenic carrier of potassium ions, is incorporated into the hydrophobic bilayer, the liposomes become strongly hyperpolarized as the potassium ions are now rapidly transported across the membrane and discharged subsequently on the external hydrophilic-hydrophobic interface. Thereby a fast, profound, 80% decrease in the emission intensity takes place since the hydrophobic cationic probes readily penetrate the lipophilic membrane in response to the strong, inside negative, transmembrane electric potential.

It is significant that the same observations can be made using pure phosphatidylcholine amphisomes. However, the addition of 25% cholesterol helps to increase the long term stability of the change in fluorescence intensity following hyperpolarization of the organized assemblies.

Implicit in the discussion above is the fact that the transmembrane partition of hydrophobic cations is potential-dependent, namely, the more negatively charged the interior of the vesicles, the more one-sided the distribution becomes towards the intravesicular space. Stated another way, when the internal compartment of the amphisomes is negatively charged, the lipophilic cations are more stable within the organizates in comparison to the external aqueous volume. Waggoner and his searchers[57] demonstrated this fact quantitatively by using human red blood cells whose steady-state membrane potential is approximately given by the Goldman-Hodgkin-Katz equation. For example, the resting transbilayer potential of red blood cells at 23°C is approximately -8 millivolts, inside negative. Thus, when DiS-C_3-(5) is added to a suspension of these globules, about 42% of the aqueous fluorophore becomes bound with the red cells. Moreover Waggoner[57] then introduced valinomycin into the red cell membrane and subsequently depleted the external concentration of potassium as to hyperpolarize the cells to an estimated -40 millivolts, inside negative; whereupon a large decrease in the fluorescence intensity occurs, as essentially all of the optical indicator cyanine dye becomes bound to the red cells.

It is significant in reference to the present investigation that Waggoner[57] suggested, by analogy with the work of Lauger[52] regarding the transbilayer distribution of lipophilic ions under the influence of a voltage gradient, that at -40 millivolts the fluorescent diS-C_3-(5) probes are most likely distributed between the exo- and endocellular surfaces with very little dye accumulated within the intracellular space, or cytoplasm. Again, self-association prevails on the cellular surfaces, owing to the high local concentration of the adsorbed dye, such that nonfluorescent aggregates bring about the decrease in emission intensity observed when the optical sensors are in the bound state.

Finally, and perhaps most importantly, as a membrane potential probe, N-methylquinolinium is superior to the cyanine dyes. The heterobicyclic structure is hydrophobic and electrostatically light, such that it similarly is able to permeate hydrocarbon bilayers. Moreover, when compared with cyanine dyes, N-methylquinolinium is a nondamaging probe, because it has a relatively small molecular skeleton. Furthermore, N-methylquinolinium is structurally rigid and therefore a more efficient emitter than most cyanine cations. In addition, N-methylquinolinium is photostable, hence not easily degraded. And last, unlike its cyanine counterparts, N-methylquinolinium is not readily adsorbed by glass, especially quartz cuvettes.

Discussion of Errors

There are some potential sources of error in the preceding analysis whose detailed description has been excluded hitherto for the sake of simplicity and clarity. These possible sources of uncertainty

are considered in this section and when appropriate they are examined in more detail in the Experimental Chapter.

Gel-chromatographic Separation

Perhaps the most intriguing characteristic of the gel-chromatographic exclusion of intervesicular N-methylquinolinium is the low recovery of the dihexadecylphosphate surfactant which, from Table 2, is about 9%. This limitation of hydrated Sephadex gels has been encountered first by Huang[46] while trying to homogenize, that is, to remove the multilamellar assemblies, from a suspension of sonicated dipalmitoylphosphatidylcholine liposomes. Huang recommended, thereby, that in order to optimize the percent recovery of the phospholipid, the hydrated stationary phase must be treated repeatedly with the phospholipid suspension and washed subsequently with the pure eluent buffer. In this manner, Huang increased the amount of recovered dipalmitoyl lecithin up to 90%.

Similarly in the present work, by repeatedly treating the gel with aliquots of the DHP suspension and by eluting subsequently with the pure phosphate buffer, the amount of recovered phospholipid, or rather surfactant, can be made to exceed 75%. It is likely that by treating the stationary phase repeatedly with the DHP suspension, the Sephadex matrix becomes saturated with the surfactant, such that the gel cannot adsorb the amphiphiles very strongly. Nevertheless, as the stationary phase is treated with aliquots of the DHP suspension, the resolving power of the gel with respect to the N-methylquinolinium solute is reduced, to the extent that the band containing the vesicles broadens and begins to overlap with that containing the free or

excluded, fluorophore. The leakage of the free N-methylquinolinium into the band containing the vesicles is troublesome especially because of the high sensitivity of the fluorometric method whereby even a small concentration of intervesicular luminophores can influence the proper assignment of the binding environments.

Dynamic Turbidimetry

When the diameter of colloidal aggregates is measured by means of dynamic light scattering, it is important to carry out the determination under conditions approaching infinite dilution, in order to minimize attractive or repulsive interactions among the particles because such intercolloidal forces perturb the diffusion coefficients of the assemblies, to generate an apparent diameter which is different from the true value. Still, in the present investigation, the vesicles whose diameter is specified in Table 2 are suspended in a very dilute medium whose total concentration of dihexadecylphosphate is 4.20×10^{-5} M and therefore much too low for vesicle-vesicle interactions to be important.

Consider, by way of an illustration, the concentration of the large, 1400 angstrom assemblies in the suspension. From the fact that these aggregates contain 1.6×10^5 DHP amphipaths, the concentration of vesicles in the gel-chromatographed eluate is simply given by

$$[\text{Vesicle}] = 4.20 \times 10^{-5}\ \text{M} \times \frac{1}{1.6 \times 10^5\ \frac{\text{monomers}}{\text{vesicle}}} = 2.6 \times 10^{-10}\ \text{M}$$

On the other hand, if the gel-chromatographed suspension contains mostly 400 angstrom vesicles, which incorporate 1.1×10^4 DHP gegenions, the concentration of these assemblies is calculated to be 3.8×10^{-9} M. Clearly, with a nanomolar, or lower, concentration of aggregates in the suspension, the influence of second order vesicle-vesicle perturbations on the diffusion coefficient can be neglected.

In contrast to the foregoing conclusion, consider the data in Table 13 which contains the hydrodynamic diameters for the liposomes in a set of gel-chromatographed suspensions, diluted serially, at constant ionic strength by using the pure phosphate buffer, pH = 7.45, as the solvent. Observe, thereby, that the measured diameter appears to increase with increasing dilution; for example, in the case of suspension II-A, the apparent hydrodynamic diameter before dilution is 1500 angstroms. On twofold dilution, the diameter appears to increase to 1620 angstroms; furthermore, upon threefold isotonic dilution, the measured diameter becomes 1670 angstroms. Now, because the suspensions are highly dilute, the apparent increase in the diameter of the liposomes upon isotonic dilution is not related to inter-particle repulsions. Instead, the increasing diameter is very likely to be another indication of the heterogeneity of the suspension. Because the intensity of the scattered, or radiated, light is dominated by the larger 1500 angstrom aggregates, the apparent diameter is strongly biased in their favor and, consequently, a serial dilution only compounds the problem by further displacing the measured diameter in favor of the larger liposomes.

Table 13. The Diameter at 25°C of Gel-chromatographed Dihexadecylphosphate Vesicles[a] Suspended in 0.0100 M NaH_2PO_4, at pH = 7.45, from Dynamic Turbidimetry.

	Average Diameter in Angstroms[b]		
	Undiluted	2-Fold Dilution	3-Fold Dilution[c]
II-A	1500	1620	1670
II-B	1470	1530	1580

[a]The conditions for the gel-chromatographic filtration are described in Table 2; the eluate contains 4.20×10^{-5} M dihexadecylphosphate.

[b]The diameter is derived from the cumulant or second moment results.

[c]Each dilution is isotonic, that is, 0.0100 M NaH_2PO_4 is used as the solvent.

Yet the accurate measurement of the diameters can be accomplished by applying a biexponential, cumulant, equation to the temporal decay of the correlation function. Here, then, is the purpose of the histogram presented in Figure 5: to give the true diameters in the bimodal distribution as equal to 400 and 1400 angstroms, respectively, in an unchromatographed suspension of vesicles whose total concentration of dihexadecylphosphate, at 1.83×10^{-4} M, is nevertheless sufficiently dilute that inter-vesicle repulsions do not alter the diffusion coefficient of the liposomes.

Dynamic and Continuous Emission Spectrometry

With regard to temporal emission spectrometry, there are two main sources of error. One refers to the fact that the instrument response function, or lamp profile, is convoluted with the fluorescence decay. Therefore, for an accurate measurement of lifetimes and a set of genuine statistical indicators, it is necessary to correct the sample decay by deconvoluting it from the characteristics of the source or lamp. Thus the profile of the lamp must be measured independently under conditions which are not the same as those employed with the sample; for instance, by using the pseudo-scatterer procedure whereby the lamp decay curve is constructed from the light radiated by a turbid colloidal suspension.

The other important source of uncertainty in dynamic fluorometry concerns the phenomenon known as the wavelength-dependent transit-time of the emission across the surface of the detecting photomultiplier tube. This phenomenon, whose presence deteriorates the quality of the statistical indicator chi-squared, has been discussed in detail by

DeSchryver and his collaborators.[58] The distortion, or correlation, which it introduces can be minimized by applying the channel displacement procedure, whereby the position of the lamp decay curve, or instrument response function, is shifted one or two channels to the right or to the left of the sample decay curve.

The channel displacement procedure is illustrated by the data in Table 14 in reference to a gel-chromatographed, unheated, suspension of dihexadecylphosphate liposomes, at 25°C. There are some important properties to be observed in this set of data. Prominent among them is the occurrence of a minimum in the value of chi-squared in terms of the displacement in the position of the instrument response function. Thus, while the initial value of chi-squared is 1.22, as the lamp curve is shifted in its position by one channel to the left of the sample decay, chi-squared decreases to its minimum value of 1.12; a further displacement of the lamp profile to the left by two and three channels merely increases the magnitude of chi-squared to 1.14 and 1.24, respectively.

While the improvement in the chi-squared parameter for the set of data included in Table 14 is comparatively mild, the procedure can be invaluable because oftentimes the fidelity of the simulation corresponding to unheated, gel-chromatographed, suspensions is unacceptable to generate values of chi-squared about 1.50 - 1.60. In these cases, the channel displacement method helps to smooth out the simulation by extracting the purely instrumental correlation from the decay of the sample.

Table 14. The Influence of the Channel Displacement Technique on the Fluorescence Decay of Intravesicular N-methylquinolinium,[a] at 25°C.

Channel Displacement[b]	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^2
0	9.42	32	3.40	68	1.22
1	9.62	30	3.63	70	1.12
2	9.80	29	3.83	71	1.14
3	9.98	27	4.00	73	1.24

[a] In 0.0100 M NaH_2PO_4 at pH = 7.45. Unheated suspension; gel-chromatographed according to the procedure described in Table 2.
[b] The lamp decay curve, or instrument response function, is displaced, by the number of channels indicated, to the left of the fluorescence or sample decay curve.

Still, another important trend to be distinguished in Table 14 refers to the monotonic drift in both the lifetimes and their corresponding mole fractions, as the lamp decay curve is displaced serially to the left of the sample decay curve. In this respect, consider the drift in the transversal or vertical lifetime T_t--its initial value is 3.40 nanoseconds, while its frequency is about 68%. On shifting the instrument response function by one channel, the lifetime increases to 3.63 nanoseconds and its mole fraction becomes 70%. An additional displacement, for a total of two channels, continues the drift in T_t to approximately 3.83 nanoseconds while the mole fraction A_t rises to 71%. Finally, when the lamp profile is shifted to the left of the sample decay by three channels, the transversal lifetime reaches 4.00 nanoseconds and its relative frequency increases to 73%.

The monotonic rise in the fluorescence relaxation constants and their corresponding frequencies is likely to be the consequence of the mathematical intricacy of the six-dimensional decay equation because in the case of a simpler monoexponential fluorescence decay, while useful to improve the fidelity of the simulation, the channel displacement procedure will not alter the unimodal lifetime.

Most importantly, in order to establish quantitative comparisons among the lifetime data obtained from a biexponential decay function--such as in the construction of an Arrhenius plot of activation

energy--consistency is of the utmost importance to the extent that the same channel displacement must be applied to all of the fluorescence decays which are being compared quantitatively; otherwise, instrumental discontinuities will interfere with any correlation of the data.

An additional limitation of dynamic fluorometry concerns the fact that because the decay function is a complicated four-parameter, biexponential polynomial, a small change in the fluorescence intensity is not readily detected. As an example, consider the emission intensity of the exovesicular N-methylquinolinium on the unheated, gel-filtered, vesicles in the presence of 0.0276 M added intervesicular thallium nitrate. By inspecting the lifetimes T_ℓ and T_t and the corresponding mole fractions A_ℓ and A_t listed in Table 6, it is apparent that on addition of thallium(I), the biexponential fluorescence decay is unchanged. Accordingly, a rapid calculation using the fluorescence data from Table 6 indicates a small, 7%, reduction in the emission intensity following addition of $T\ell NO_3$.

By contrast, the Stern-Volmer titration obtained in the continuous emission mode, and presented in Figure 10, shows a 17% reduction in the fluorescence intensity when 0.0276 M $T\ell NO_3$ is added into the unheated, gel-chromatographed suspension of vesicles. It is clear, therefore, that the steady or continuous emission fluorometer provides the more sensitive tool for measuring a small change in the fluorescence intensity. Conversely, dynamic fluorometry with its more complicated decay function cannot be used reliably for the quantitative measurement of a small change in the fluorescent emission. Observe,

furthermore, that the two-channel displacement in the instrument response function, which introduces a systematic increase in both the relaxation constants and their respective frequencies, does not apply in the continuous emission mode.

Finally, James and Ware[59] have pointed out that a biexponential distribution in the fluorescence lifetime can be an indication of a much more complex, underlying, set of relaxation constants. They demonstrated this fact by calculating the fluorescence decay for a single, yet broad, gaussian distribution in the lifetime and observed, thereby, that when the standard deviation of the mean--or the width of the distribution--exceeds 15%, the fluorescence can be analyzed adequately in terms of a double exponential decay that generates, invariably, a set of two distinct fluorescence time constants with their respective preexponential factors. Ware and James,[59] therefore, warn that the biexponential analysis alone cannot be used to prove the existence of discrete binding environments for the two component lifetimes.

Moreover, Ware and his collaborators[59] suggest that in order to detect the underlying complexity by means of the usual fidelity indicators, of which chi-squared is one, it is necessary to count a very large number of photons, in the range between 50×10^3 and 500×10^3, at the channel of maximum intensity. This large storage capability, Ware indicates, is not possible with most instruments; nor for that matter, are most dynamic fluorometers--especially those which utilize a gas-discharge lamp as the excitation source--able to

function properly for the extended period of time required to count such a large number of pulses.

Alternatively, he suggests the use of independent experimental evidence, of the kind employed in this investigation, as the only satisfactory means to assign discrete binding environments to the component lifetimes recovered from a biexponential fluorescence decay.

Endo- and Exovesicular Adsorption

Regarding the identification of the binding environments, it is the usual practice to compare the spectroscopic properties of the bound luminophore with those observed in a series of organic solvents having different dipole moments. Menger,[60] however, has expressed reservations concerning this stratagem, because it is difficult to devise a medium which will solvate an aromatic ring with water on one side and with hydrocarbon on the other--as is likely to be the case when N-methylquinolinium is bound to the water-hydrocarbon interfaces of dihexadecylphosphate vesicles. Moreover, lifetime measurements in organic solvents are not carried out in the present work because the cationic excited state manifold of N-methylquinolinium is a good electron acceptor. Hence, in the presence of a potentially electron-donating solvent like an alcohol, acetonitrile, or dioxane, deactivation may take place because of the favorable reduction potential of the electronically excited acceptor, rather than as a consequence of a change in the polarity of the medium.[61]

Instead, the relaxation constant of N-methylquinolinium is measured in the presence of sodium dodecylsulfate micelles because their matrix has been determined to consist of water-hydrocarbon interfaces

which simulate faithfully the residence sites of N-methylquinolinium adsorbed longitudinally on the surfaces of dihexadecylphosphate vesicles. In addition, the use of an increasing concentration of sodium perchlorate, to observe the influence of a rising polarity on the relaxation constant, circumvents the complications introduced by possible deactivating solute-solvent associations, since $NaClO_4$ represents a strong, yet otherwise inert electrolyte.

At last, in contrast to its fluorescence relaxation constant, the position of the emission maximum of N-methylquinolinium at 400 nanometers is not particularly sensitive to incorporation of the probe within the organized assemblies which would otherwise be useful to further characterize the microtopography of the binding sites.

Now, concerning another aspect of adsorption on dihexadecylphosphate vesicles, the calculation of the area occupied by the endo- and exovesicular surfaces is based on the external diameter of the assemblies, as determined from dynamic turbidimetry, and assumes that the vesicles are spherical with a bilayer length about 50 angstroms.[4] In this respect, the shape of DHP liposomes is not without controversy.

Herrmann and Fendler,[62] for instance, are the chief proponents of the prolate ellipsoidal DHP assembly; this means that the vesicles are shaped in the form of discs which they suggest to have a longitudinal axis about 2000 angstroms and a transversal girth approximately equal to 100 angstroms. Still, Furlong and his associates[21] have criticized such an assignment on the basis that it would cause the aggregates to possess a binary diffusion coefficient, that is, one that contains a translational as well as a rotational component,

both of which would contribute to generate a multiexponential autocorrelation equation in dynamic turbidimetry. Instead, Furlong indicates that the analysis by Herrmann and Fendler[62] is probably complicated by a heterogeneous distribution in the diameter of the vesicles in their suspension, and that the spherical shape represents the better model for the DHP assemblies.

Consider now, the calculation of the initial distribution of N-methylquinolinium along the endovesicular ridge. In order to reproduce, or simulate, the frequencies of the two component lifetimes in the gel-chromatographed, unheated, assemblies it is necessary to adjust, or optimize, the surface area per adsorbed N-methylquinolinium to about 200 angstroms squared. Again, this assignment is substantiated by the work of Furlong and his colleagues[21] who measured the surface area of the geometrically equivalent, bihexagonal, methylviologen dication to be equal to 185 angstroms squared, by experiment from its adsorption on inorganic oxides and, most importantly, on dihexadecylphosphate vesicles at pH 8.0.

Clearly, the field of surface chemistry is sufficiently broad that a value may be found somewhere in the literature which will corroborate the area per adsorbed fluorophore suggested by the optimization procedure above. Therefore, the calculation of the endovesicular fluorophore distribution in the unheated, gel-filtered, assemblies would remain a mathematical curiosity were it not for the fact that the same 200 angstroms squared can be used to calculate accurately the extent of transmembrane permeation of N-methylquinolinium at 45°C; according to its equilibrium distribution between the endo- and exovesicular surfaces.

A further relevant question concerns the initial concentration of the probes adsorbed on the external surface of the unheated, gel-filtered, vesicles. For instance, the 17% decrease in the emission intensity on addition of 0.0276 M intervesicular thallium(I) seems to indicate that a substantial mole fraction of N-methylquinolinium is bound to the external, or exovesicular, surface. In this regard, an equation derived by Almgren[24] comes to mind, which he has employed to estimate the fraction of tris(2,2'-bipyridine)ruthenium(II) bound to the external surface of dihexadecylphosphate assemblies, by measuring the decrease in the steady state emission on addition of intervesicular methylviologen quenchers.

Yet, the equation of Almgren[24] cannot be applied to determine the initial concentration of exovesicular N-methylquinolinium in the present investigation, because there exists a distribution of lifetimes such that a decrease in the continuous emission, as a result of the selective deactivation of the external, longitudinal, adsorbates, must be corrected for the fact that the separate components contribute differently to the observed fluorescence; since the independent states emit light with varying intensity, in direct proportion to their radiative lifetime.

Moreover, Almgren[24] designed his equation to be utilized under such conditions that the ratio of the concentration of DHP with respect to that of the quencher is at least 20:1, at the highest concentration of added deactivator. With these conditions, Stern-Volmer dynamics apply because the methylviologen quencher does not accumulate on the outer surface of the vesicles. Thereby, the frequency

of collisional deactivation is proportional to the concentration of the cationic quenchers introduced into the aqueous phase.

By contrast, in this investigation the lowest concentration of added thallium(I), at 3 millimolar, is many times greater than the concentration of dihexadecylphosphate, in the suspension, at 4.20×10^{-5} M. Consequently, while Stern-Volmer dynamics are still clearly obeyed, the frequency of collisional deactivation will be determined by the concentration of thallium(I) on the outer surface of the vesicles which will be greater than its concentration in the bulk aqueous phase; thus precluding the direct application of the Almgren equation. Incidentally, the large excess of the cationic thallium(I) quencher with respect to the concentration of dihexadecylphosphate is not troublesome because, at ambient temperature, the organizates are quite impermeable to hydrophilic cations, especially univalent metal ions.[24]

In any event, returning to the original problem, it is possible to show that the initial concentration of exovesicular N-methylquinolinium must be small from the fact that when an incubated suspension is treated with sodium dithionite, whereby the exovesicular probes are reduced, the recovered frequency distribution is identical to that found with the unheated, gel-filtered, suspension.

Finally, it should be mentioned that ground state association between the heterocyclic cations must be absent because the absorption spectrum of N-methylquinolinium is independent of concentration.

Epilog

The microtopography of sodium dihexadecylphosphate vesicles, including the distribution of the N-methylquinolinium probes between

the internal and external walls, is reminiscent of the aforementioned thylakoid assemblies which are found within the chloroplasts of green plants.

By way of an illustration, the internal volume of a thilakoid vesicle is approximately equal to 1×10^{-15} cm^3, as compared with 1.4×10^{-15} cm^3 for the intravesicular volume of a spherical DHP organizate whose diameter is about 1500 angstroms. Similarly, even though the membrane of the thilakoids has a high content of protein, which accounts for its granular appearance in freeze-fracture electron micrographs, the internal and the external surfaces are both negatively charged because of the anionic glycolipids anchored along the water-hydrocarbon ridges. The charge density is similar on both surfaces, about 1 - 3 microcoulombs per centimeter squared, corresponding to one anionic site for every 500 - 1600 angstroms squared.[10]

Furthermore, the thilakoid membrane contains about 1×10^5 chlorophyll molecules. In comparison, a DHP organizate whose external diameter is about 1500 angstroms contains nearly 9.0×10^4 N-methylquinolinium luminophores. Of the 1×10^5 chlorophyll molecules in the thilakoid structure, a portion is adsorbed on the external surface in a photosynthetic unit described as system I--such adsorbates are reminiscent of the longitudinal, exovesicular N-methylquinolinium probes on the DHP vesicles. Now, the remaining chlorophyll molecules not associated with system I, are assembled as part of another

photounit, system II, which is largely but not entirely, buried within the hydrophobic matrix of the thilakoid membrane, in proximity to the inner aqueous compartment--such adsorbates are, in turn, reminiscent of the transversal or intercalated endovesicular N-methylquinolinium probes within the DHP colloids.[10]

The preceding similarities between the thilakoid assemblies and the sodium dihexadecylphosphate vesicles illustrate the fact that the latter organizates appear to hold some promise in the field of light transduction and man-made, artificial, photosynthetic devices.

Alternatively, the presence of endovesicular intercalation in dihexadecylphosphate vesicles might be important in the field of ambient temperature phosphorometry; since below the thermotropic phase transition, the hydrocarbon bilayer becomes more rigid to provide, perhaps, an alternative matrix to the use of supercooled alcoholic glasses.

Concerning the use of N-methylquinolinium as a molecular voltmeter in membranogenic fluorometric potentiometry, even though the technique has been known about two decades, additional fundamental investigations are necessary to standardize the changes in emission intensity with respect to the magnitude of the transmembrane potential.

Finally, looking ahead in the next chapter, a detailed mechanism for the transmembrane transport of N-methylquinolinium, at 45°C, is to be presented which is based on the polarization of the dihexadecylphosphate membrane resulting from the outward diffusion of N-methylquinolinium along its concentration gradient. As it turns out, the

transbilayer electric field--inside negative--created in this manner, provides the potential gradient down which sodium cations are observed to cascade.

CHAPTER III
DYNAMICS OF TRANSLOCATION OF N-METHYLQUINOLINIUM ACROSS DIHEXADECYLPHOSPHATE VESICLES

The net diffusion of a solute, s, across a semipermeable barrier, or membrane, may be described in analogy with a chemical reaction, in terms of two opposing unidirectional components

$$J_{NET} = \vec{J}_s - \overleftarrow{J}_s \tag{16}$$

where the flux $\vec{J}_s$ in mole cm^{-2} sec^{-1} from some compartment, a, is proportional to the concentration C_s^a of the solute on that side of the boundary

$$\vec{J}_s = P_s \cdot C_s^a \tag{17}$$

while the flux in the opposite direction, $\overleftarrow{J}_s$, from compartment b is similarly proportional to the concentration of the solute in that cell

$$\overleftarrow{J}_s = P_s \cdot C_s^b. \tag{18}$$

Here the proportionality constant, P_s, is the permeability, usually expressed in cm/sec, which is an intrinsic property of the semipermeable barrier. The permeability is analogous to the more familiar rate constant of a chemical reaction except that the magnitude of P_s is independent of the direction of flow.

The net diffusion, or flux, across the membrane from compartment a into b takes on the form

$$J_{NET} = P_s(C_s^a - C_s^b). \tag{19}$$

Furthermore, when the volume in one of the cells, for example in b, is very large such that C_s^b remains small during the diffusion process, or

equivalently, in the absence of efflux from cell b, equation (19) is simplified

$$J_{NET} = P_s \cdot C_s^a. \tag{20}$$

Now, the rate with which the concentration C_s^a decreases may be expressed in the form

$$- \frac{dC_s^a}{dt} = J_{NET} \cdot \left(\frac{A}{V}\right) \tag{21}$$

where A represents the area of the membrane in contact with the compartment and V stands for the volume of the cell. Combining equations (20) and (21), the result obtains

$$- \frac{dC_s^a}{C_s^a} = P_s \frac{A}{V} dt \tag{22}$$

which on integration within the boundary conditions

$C_s^a = C_s^a(0)$ at $t = 0$ and

$C_s^a = C_s^a(t)$ at time t

yields the usual first order exponential rate equation

$$C_s^a(t) = C_s^a(0) \exp(-kt) \tag{23}$$

where k represents the rate constant for diffusion, or translocation, across the semipermeable barrier, or membrane. Observe that the rate constant k is clearly defined as

$$k = P_s \frac{A}{V}. \tag{24}$$

On the other hand, the permeability, P_s, depends on the diffusion coefficient, D, of the solute across the membrane; on the thickness,

d, of the membrane; and upon the partition coefficient, K, of the permeant between the aqueous and the membranogenic domains, according to the relationship

$$P_s = \frac{D}{d} K \tag{25}$$

Equation (25) implies that the permeant species is distributed between the aqueous phase and the hydrophobic membrane in a rapid equilibrium such that the rate-limiting step, or energy barrier, is associated with diffusion of the solute across the membrane proper. Nevertheless, in the case of a strongly hydrated specie, entry into the hydrophobic bilayer is slow compared with diffusion across the membrane. Hence, the rate-limiting step, or energy barrier, is associated with the translocation step from the aqueous phase into the hydrocarbon membrane.

While N-methylquinolinium is a hydrophobic permeant, it also represents a charged specie and therefore electrical neutrality must be maintained in order for net transmembrane diffusion to take place. The condition of electrical neutrality implies that translocation of N-methylquinolinium must require codiffusion of an anion, or alternatively, counter-diffusion of a cation. Thereby, it is apparent that the nature of the energy barrier associated with the transmembrane flux of the fluorophore will be determined by the mode in which the heterocyclic cations migrate across the hydrocarbon bilayer.

Codiffusion of Anions

Consider first, the mode of translocation whereby N-methylquinolinium diffuses across the membrane in the form of an ion pair. In

this respect, Parsegian[63] has pointed out that the pair of opposite charges will diminish or cancel each other when there exists a covalent association between the two counterions, in which case the solubility of the permeant species in the nonpolar membrane will increase, in proportion to the strength of the covalent interaction. Hence, the transfer from the polar aqueous phase into the hydrophobic membrane will be a fast event, and, consequently, diffusion across the hydrocarbon bilayer will represent the rate-limiting step. For example, Haynes[64] has measured the rate of transmembrane migration of the sodium salt of 1-anilino-8-naphthalenesulfonate, ANS^-, across neutral phosphatidylcholine vesicles and found that the translocation takes place by means of an ion-pair mechanism. Moreover, it is an extremely fast process, whose time domain is on the scale of milliseconds. Such a fast rate of permeation may be expected from the fact that the low dielectric constant within the membranogenic environment will cause the sodium counterion to coordinate strongly with the sulfonate group in the ANS^- skeleton to lower the charge density of the ion-pair and increase its membranogenic solubility.

In respect to N-methylquinolinium, there are several intravesicular counterions available for transmembrane codiffusion, such as mono- and dihydrogen phosphate, from the buffer in the medium, as well as perchlorate, from the salt of N-methylquinolinium. Still, the heterocyclic cation does not bind covalently with its counterions, and, thus, the charge density of the components in the pair remains unchanged. Furthermore, Parsegian[63] has demonstrated that the energy of an ion pair, held together within a layer of hydrocarbon

by a purely electrostatic attraction, is determined by the respective ionic radii. For instance, when the ionic radii are identical and the internuclear distance is at its minimum, the energy of the ion pair is identical to the energy of formation, or charging work, calculated when each ion is suspended separately in the dielectric medium. On the other hand, when the radii of the component ions are very different, and the internuclear distance is at its minimum, the charging work required to create the electrostatic aggregate is determined by the energy of the smaller, more electrostatically dense, component particle. Thus, in the case of N-methylquinolinium, which will not bind covalently with its counterions, mono- and dihydrogen phosphate, or perchlorate, the energy required to generate a contact ion-pair within a layer of hydrocarbon is dictated by the more hydrophilic, electrostatically dense, anions.

The charging work or free energy of activation, $\Delta G^{\neq}$, required to transfer a fully hydrated ion from the bulk aqueous phase into the hydrocarbon membrane depends upon the radius, r, of the charged particle, and the difference in the dielectric constant between the two media, according to the Born equation[65]

$$\Delta G^{\neq} = \frac{N_A e^2}{8\pi \epsilon_o r}\left(\frac{1}{\epsilon_m} - \frac{1}{\epsilon_w}\right) \qquad (26)$$

in which ε_m and ε_w represent the dielectric constants in the membrane and the aqueous phase, respectively; ε_o represents the permittivity of vacuum, at 8.85×10^{-12} coulomb^2 $\cdot$ newton^{-1} $\cdot$ m^{-2}, while N_A stands for Avogadro's number. The constant e refers to the electronic charge, at 1.602×10^{-19} coulombs, such that the free energy of activation has units of joules per mole.

Since from equation (26), the free energy of activation for transposition into the nonpolar membrane is inversely proportional to the radius of the ion, it is simple to design a stratagem to investigate the possibility that transmembrane permeation of N-methylquinolinium may require codiffusion of an anion. Certainly, introducing a comparatively large anion within the intravesicular volume will lower the free energy of activation for interfacial transfer of the contact, or electrostatically coupled, ion-pair, and therefore result in a faster rate of transmembrane diffusion. Accordingly, the toluenesulfonate counterion may be considered to be somewhat larger than the phosphate or perchlorate anions because of its tolyl functional group.

As indicated in Chapter II, this anion is readily incorporated into the dihexadecylphosphate vesicles by employing an aqueous solution containing the toluenesulfonate salt of N-methylquinolinium,

rather than the perchlorate compound, in the sonification procedure. Now then, the data in Table 15 illustrate the results obtained from a permeation experiment, at 45°C, for assemblies that contain toluenesulfonate. A comparison against the extrusion of N-methylquinolinium in the absence of intravesicular toluenesulfonate, listed earlier in Table 7, indicates that the presence of the latter counterions changes neither the rate nor the extent of transmembrane diffusion of the fluorophore. To illustrate, from Table 15, the unheated suspension yields the longitudinal time constant, T_{ℓ}, at 7.31 nanoseconds whose frequency, A_{ℓ}, is approximately 23%, while the transversal time constant, T_t, at 3.30 nanoseconds has a frequency about 77%. Subsequently, on incubation for 20 hours at 45°C, the mole fraction for longitudinal adsorption increases to 42%. Further incubation for 40 hours, at the same temperature, generates a frequency of longitudinal adsorption, A_{ℓ}, about 47%. Furthermore, after 60 hours of incubation at 45°C, the mole fraction, or frequency, A_{ℓ}, is 54%. Finally, on prolonged heating, at 45°C, A_{ℓ} reaches its maximum value at just about 60%. Observe hereby, that both the rate at which A_{ℓ} increases and its equilibrium value, or the extent of vertical translocation, are the same within the experimental margin as for the vesicles which contain only phosphate and perchlorate as the intravesicular counterions. To

Table 15. The Rate of Exovesicular Discharge of N-methylquinolinium from Gel-chromatographed[a] Dihexadecylphosphate Vesicles, at 45°C and pH = 7.45, in the Presence of 7.00 x 10^{-3} M Intravesicular Toluenesulfonate.[b]

Incubation[c] time, hr	T_ℓ, ns[d]	A_ℓ, Percent	T_t, ns	A_t, Percent	χ^2[e]
0	7.31	23	3.27	77	1.07
20	8.25	42	3.55	58	0.937
40	8.34	47	3.77	53	1.03
60	8.08	54	3.49	46	1.05
80	8.28	52	3.89	48	1.04
100	7.86	59	3.46	41	0.937

[a]The buffer is made up of 0.0100 M NaH_2PO_4; the conditions for the gel-chromatographic filtration are as described in Table 2.

[b]The vesicles are prepared by sonication in the presence of 7.0 millimolar N-methylquinolinium toluenesulfonate, instead of 10 millimolar N-methylquinolinium perchlorate; and gel-chromatographed subsequently to exclude the intervesicular fluorophore.

[c]After incubation at 45°C, the suspension is allowed to equilibrate to 25°C, for about 30 minutes, and then the fluorescence decay is measured.

[d]The relaxation constant of N-methylquinolinium in the presence of 7.0 x 10^{-3} M sodium tosylate, at pH = 7.45, is 6.21 nanoseconds.

[e]The lamp calibration curve, or instrument response function, is displaced one channel to the left of the sample decay curve.

suggest that the mode of transmembrane extrusion of the fluorescent probes does not depend upon the translocation of an electrostatically bound ion-pair. Incidentally, in the presence of intravesicular toluenesulfonate, the longitudinal relaxation constant, T_{ℓ}, is found to increase across the incubation period, to contrast with the fluorescence decays measured previously, from about 7.3 nanoseconds in the unheated suspension, up to approximately 8.0 nanoseconds, after prolonged incubation at 45°C. The slight but monotonic increase in T_{ℓ} likely results from the absence of toluenesulfonate in proximity to the external surface of the vesicles; as the gel-chromatographic filtration effectively excludes the intervesicular deactivator.

A similar conclusion concerning the absence of translocation of an ion-pair may be reached from a different perspective. As transbilayer migration of the pair of counterions represents an electrically silent, or electroneutral Donnan process, the dihexadecylphosphate assemblies must remain electrically balanced during extrusion of the fluorophore. Hence, the fluorescent cations ought to be released completely, because of their exceedingly large concentration gradient. To illustrate, recall the earlier calculation concerning a suspension of 1400 angstrom vesicles, at 4.20×10^{-5} M dihexadecylphosphate, in which the intervesicular volume is estimated to be 5000-fold greater than the intravesicular

space. Moreover, in a suspension of 400 angstrom organizates, at 4.20×10^{-5} M DHP, the intervesicular volume is a staggering 30,000-fold greater than the intravesicular space. Clearly therefore, under the influence of such a strong, outwardly-directed, concentration slope, the discharge of the fluorescent cations into the bulk aqueous phase ought to proceed to completion, which would invariable result in a single exponential fluorescence decay with a lifetime equal to the relaxation constant of the free fluorophore in the pure phosphate buffer, that is, 8.88 nanoseconds. Nevertheless, the fluorescence decay of the gel-chromatographed suspension of vesicles is observed to remain bimodal even after prolonged incubation at 45°C; again, to suggest that the mode of transmembrane extrusion of N-methylquinolinium likely does not require the electrically silent translocation of an ion-pair.

Finally, it is easy to exclude the mechanism concerning the transmembrane migration of an ion-pair from an inventory of the ionic composition of the intravesicular medium. Consider first, the case of the vesicles whose diameter is 400 angstroms. Because the anionic monomers along the inner monolayer associate predominantly with the more hydrophobic heterocyclic cations, the internal concentration of sodium ions may be taken equal to 0.0200 M, or the same as in the hydrating medium, or buffer, used to prepare the assemblies. Under these conditions, that is, with 0.0200 M intravesicular Na^+, a 400 angstrom organizate is calculated to contain 170 sodium cations within its aqueous pocket. By the principle of macroscopic electrical neutrality, there must be 170 free, readily mobile, negative charges

within the intravesicular volume, provided by mono- and dihydrogen phosphate, or perchlorate ions. Then again, by macroscopic electroneutrality, the maximum number of N-methylquinolinium fluorophores that will diffuse across the membrane is 170, as there is only that number of free, readily mobile, intravesicular anions with which to form contact ion-pairs. Yet about 2500 N-methylquinolinium ions are observed to diffuse across the membrane in order to saturate the exovesicular surface; to explain the extent of permeation observed on prolonged incubation at 45°C.

Consider alternatively, the assemblies whose diameter is about 1400 angstroms. When prepared in the presence of 0.0200 M Na^+, each vesicle is calculated to contain 14,000 sodium cations within its aqueous pocket. Again, the principle of macroscopic electrical neutrality indicates that there must be 14,000 free, readily mobile, negative charges within the intravesicular volume; hence, according to the ion-pair mechanism, the maximum number of heterocyclic cations that will diffuse across the membrane is 14,000. Yet 31,000 N-methylquinolinium probes are observed to diffuse across the membrane, in order to saturate the exovesicular surface; to explain the extent of translocation observed on continued incubation at 45°C. It is clear, thereby, that transmembrane diffusion of ion-pairs represents an inefficient mechanism for the permeation of N-methylquinolinium, because the number of free, readily translocated, intravesicular anions is not sufficient to account for the extent of permeation observed by experiment, on continued heating at 45°C. In conclusion, the mode of extrusion of the fluorescent cation does not depend upon the translocation of a pair of oppositely charged ions.

Counterdiffusion of Cations

An alternative mechanism for the net transport of N-methylquinolinium, according to the law of macroscopic electroneutrality, requires that diffusion of the biheterocyclic ions, from the endovesicular surface on to the exovesicular reef, generates a current of oppositely directed cations, or counterdiffusion. Accordingly, there are only two cations present in the extravesicular volume which may participate in antiparallel diffusion, namely, hydrogen and sodium ions.

Antiparallel diffusion of hydrogen ions.--A working estimate of the pKa of dihexadecylphosphoric acid may be obtained about 3.5, by comparison with the acid dissociation constant of phospholipid molecules like phosphatidic acid.[4] Therefore, at pH = 7.45, the concentration of hydrogen ions on the vesicular surfaces is very small. Although Nichols and Deamer[66] have observed fast transbilayer diffusion of hydrogen ions, which they attribute to the ability of H^+ to migrate by means of hydrogen bond exchange, it is not likely that a proton countercurrent will influence the rate of translocation of N-methylquinolinium, because of the predominant presence of sodium cations on the exovesicular surface.

It is easy to demonstrate the preceding conclusion. Since transbilayer diffusion of H^+ represents a fast process, then at low intervesicular concentration of H^+, the rate of proton translocation, and, thereby, the countertransport of N-methylquinolinium, depends on the frequency with which the vesicles encounter hydrogen ions, and therefore, upon the intervesicular pH. Accordingly,

Table 16 contains the permeation data in terms of the incubation time, at 45°C, for a suspension of gel-chromatographed vesicles in phosphate buffer, at pH 9.05. A direct comparison with the permeation data listed in Table 7, for the suspension of chromatographed assemblies at pH = 7.45, indicates that the rate of transbilayer diffusion of N-methylquinolinium is independent of the hydrogen ion concentration in the medium. To illustrate, from the data in Table 16 corresponding to the pH = 9.05, the longitudinal lifetime, T_{ℓ}, in the unheated suspension is about 8.60 nanoseconds and the corresponding frequency, A_{ℓ}, approximately 26%; while the transversal relaxation constant, T_t, is 3.62 nanoseconds and the respective frequency, A_t, about 74%. Upon incubation at 45°C, the usual temporal evolution of the permeation is observed. For example, after 20 hours at 45°C, the longitudinal frequency, A_{ℓ}, has increased to 44%; after 40 hours of incubation at the same temperature, A_{ℓ} is about 46%; and after 60 hours, A_{ℓ} increases to approximately 57%; finally, upon continued heating, the magnitude of the longitudinal frequency reaches its maximum value near 60%. Certainly, the temporal evolution of the permeation at pH = 9.05 is the same, within the experimental margin, as observed in the case of the reference suspension, whose pH is 7.45. Hence, transbilayer migration of N-methylquinolinium likely does not require counterdiffusion of hydrogen cations.

Antiparallel diffusion of sodium ions.--According to the preceding analysis, the translocation of the cationic luminophores requires neither the comigration of a negative charge nor the countermigration of hydrogen cations. Consequently, the outwardly-directed permeation

Table 16. The Rate of Exovesicular Discharge of N-methylquinolinium from Gel-chromatographed Dihexadecylphosphate Vesicles at 45°C, in 0.0100 M NaH_2PO_4 at pH = 9.05[a].

Incubation[b] time, hr	T_ℓ, ns[c]	A_ℓ, Percent	T_t, ns	A_t, Percent	χ^2[d]
0	8.60	26	3.62	74	1.23
20	8.22	44	3.79	56	1.22
40	8.46	46	4.12	54	0.976
60	8.08	57	3.78	43	0.983
80	8.13	58	3.88	42	1.19
100	8.13	61	4.02	39	1.04

[a]The conditions for the gel-chromatographic filtration are as described in Table 2, except that 0.0100 M NaH_2PO_4 at pH = 9.05 is used to hydrate the gel-matrix, and also as the eluent during the separation. The sodium ion concentration in the phosphate buffer, at pH = 9.05, is about 0.0200 M.

[b]After incubation at 45°C, the suspension is allowed to equilibrate to 25°C, for about 30 minutes, and then the fluorescence decay is measured.

[c]The relaxation constant of N-methylquinolinium, in the presence of 0.0100 M NaH_2PO_4, at pH = 9.05, is 7.80 nanoseconds.

[d]The lamp calibration curve, or instrument response function is displaced one channel to the left of the sample decay curve.

of N-methylquinolinium must require the counterdiffusion of sodium ions.

The existence of the sodium ion countercurrent has a profound implication with respect to the rate of permeation of N-methylquinolinium, because the former are electrostatically much denser than the flat, charge-delocalized, biheterocyclic cations. Thus, because of their higher charge density, sodium ions have a much lower membranogenic solubility, and, consequently, the rate of transmembrane diffusion of N-methylquinolinium is controlled by the much slower counterflow of sodium ions. Clearly, the cationic fluorophores provide an optical indicator with which to signal, and measure, the rate of inwardly-directed translocation of Na^+.

Since the diffusion of N-methylquinolinium is controlled by the slower antiparallel flow of sodium ions, the existence of the Na^+ countercurrent may be demonstrated by measuring the rate constant of permeation reported by N-methylquinolinium, and comparing its value against the rate coefficients given elsewhere in the literature, for diffusion of sodium ions across the membrane of unilamellar vesicles.

Still, a comparison with the permeability constants given in the literature, for translocation of sodium ions across hydrophobic bilayers, presents a delicate problem, as follows:

a. The permeability constant of sodium ions is strongly dependent on the temperature.

Below the lipid bilayer phase transition, the membrane of vesicles is extremely impermeable to strongly hydrophilic matter, such as sodium ions, because the semicrystalline state in which the bilayer

exists excludes other matter from its matrix, especially charged solutes. By contrast, at the phase transition temperature, the permeability of the bilayer increases very strongly as it enters the fluid, or liquid-crystal, state. To illustrate this phenomenon, observe that the permeability of hydrophobic bilayers, with respect to sodium ions, increases drastically, by about 100-fold, at the phase transition temperature.[13,35,67]

With respect to the phase transition temperature, T_c, of dihexadecylphosphate, Kunitake and his coworkers[68] have reported a value of 66°C for a suspension of the sodium salt of DHP in deionized water. Nevertheless, the comparatively high value of T_c provided by Kunitake[68] does not correspond with the observations in the literature concerning the efficient permeation of dihexadecylphosphate vesicles at temperatures considerably lower than 66°C. For example, Fendler[50] indicates that transmembrane diffusion of the hydrophilic methylviologen dication is rapid at 30°C and neutral pH. Similarly, Furlong and his associates[51] have observed the onset of transmembrane migration about 35°C for both methylviologen and tris(2,2'-bipyridine)-ruthenium(II). Furthermore, in the present work, transmembrane diffusion of N-methylquinolinium is observed to take place at 45°C.

On the other hand, simultaneously with the work of Kunitake,[68] Kano and his collaborators[69] employed the nonpolar fluorescent probe 1,3-di(1-pyrenyl)propane, abbreviated as P_3, to determine the phase transition temperature of sonicated dihexadecylphosphate vesicles. P_3 is a sensitive probe to determine the bilayer phase transition

temperature, because the ratio I_E/I_M corresponding to the fluorescence intensity of the excimer relative to that of the monomer form of the bichromophore, increases abruptly at the thermotropic phase transition. Still, when P_3 is incorporated into the bilayer of small, sonicated, DHP vesicles at pH = 8.0, there takes place a gradual increase in the ratio I_E/I_M, starting about 35°C. This observation suggests that the onset of the phase transition of sonicated dihexadecylphosphate vesicles, at pH = 8.0, takes place about 35°C, and extends on a range where liquid and solid domains coexist.

Now, Papahadjopoulos[35] has pointed out that the existence of liquid and solid domains drastically increases the permeability of the hydrophobic bilayer, especially with respect to the translocation of ions, because such clusters represent regions of microscopic disorder across whose boundaries enhanced diffusion takes place. For instance, with his associates, Papahadjopoulos has invoked the presence of solid and liquid domains to account for the discontinuous increase, about 100-fold, in the permeability of phospholipid bilayers, with respect to sodium ions, at the thermotropic phase transition. It follows that the observation by Kano and his coworkers[69] regarding the broad range in the phase transition temperature of sonicated DHP vesicles, which suggests the coexistence of partially solid and liquid clusters, starting about 35°C, corresponds well with the enhanced ionic permeability observed near that temperature in the cases of N-methylquinolinium, tris(2,2'-bipyridine)ruthenium(II) and, particularly, in the case of the more hydrophilic methylviologen dication.

The apparent difference between the observations of Kunitake,[68] on the one hand, and the results of Kano,[69] on the other, is useful because it illustrates the fact that preparation of the suspension, especially the extent of sonification, alters the physicochemical state of the vesicles. As an example, it is well known that multilamellar and sometimes large unilamellar vesicles yield extremely narrow thermotropic transitions, whose width is commonly less than one degree; as expected from a synchronous event in which a large number of phospholipid molecules cooperate to enter the liquid-crystal, or fluid state. By comparison, when the assemblies are sonicated well, to produce small, single bilayer vesicles, the midpoint of the phase transition is lowered; the enthalpy of the phase transition decreases, sometimes as much as 50%, such that the thermotropic change becomes broad.[70]

Consider, for instance, the work of Mabrey and Sturtevant,[71] concerning the reversible phase transitions of multilamellar and sonicated vesicles of dimyristoylphosphatidylcholine--a phospholipid whose hydrophobic skeleton contains 14 carbon atoms. By using differential scanning calorimetry, they observed that the multilamellar assemblies have a sharply defined phase transition at 24.3°C. In comparison ,the thermogram obtained using the sonicated assemblies is quite different--the midpoint of the phase transition is lowered to 19.5°C, while the temperature range is very broad and extends from 10°C up to approximately 30°C.

Mabrey-Gaud[70] has attributed the broad phase transition of the small unilamellar vesicles to the configurational strain among the

phospholipid monomers which results from the inherently higher radius of curvature in small single bilayer vesicles. Consequently, the packing density of the phospholipid monomers is altered, as demonstrated from the fact that the outer monolayer of small unilamellar vesicles contains nearly twice as many phospholipid molecules as the inner monolayer. Furthermore, a change in the packing density of the monomers reduces the size of the cooperative unit undergoing the phase transition. For instance, in the case of multilamellar or large unilamellar liposomes made up of dimyristoylphosphatidyl lecithin, Mabrey-Gaud,[70] using differential scanning calorimetry, has shown that the unit undergoing the cooperative transition contains about 330 phospholipid molecules. On the other hand, Gruenewald and his collaborators[72] have determined that when the diameter of the DMPC vesicles is less than 800 angstroms, the number of phospholipid monomers in the cooperative cluster is reduced to 100. Clearly then, for small unilamellar liposomes, entrance into the fluid or liquid-crystal state is less likely to be concerted, hence domain formation, or coexistence of liquid and solid clusters, takes place, such that the temperature range for the transition is broadened.

b. The microtopography of the bilayer regulates the translocation of sodium ions.

For vesicles of comparable dimensions, the packing density and alignment of the surfactant monomers is determined by two parameters, namely, the length of the hydrocarbon chains and the surface area of the headgroup anchors. Thus, an adequate comparison against the rate of translocation of sodium ions across hydrophobic bilayers reported

in the literature, requires the selection of a reference phospholipid whose alkyl chain length and headgroup area are nearly the same as for the dihexadecylphosphate gegenion.

Consider therefore, the hydrocarbon chain length and headgroup area of dipalmitoylphosphatidylcholine--the most commonly used phospholipid in the preparation of liposomes. While superficially the neutral DPPC phospholipid appears different from dihexadecylphosphate, the lecithin phospholipid has at least 15 carbon atoms in its hydrophobic chains. Further, at the phase transition temperature, 41°C, and above, the PC headgroup is measured to occupy an area equal to 70 angstroms squared. Thus, at neutral pH, above 41°C, the areas occupied by the DPPC and DHP headgroups are the same, both at 70 angstroms squared. Incidentally, the area taken up by the DHP headgroup is not very sensitive to the temperature, because the predominant repulsive forces among headgroups are electrostatic, rather than van der Waals interactions, as in the case of phosphatidylcholine. Hereby, in the fluid or liquid-crystal phase, above 41°C, dihexadecylphosphate and dipalmitoylphosphatidylcholine vesicles, having similar dimensions, possess bilayers in which the packing density and alignment of the monomers are comparable, to indicate that the assemblies ought to have similar sodium ion permeabilities.

c. The net extrusion of N-methylquinolinium takes place with simultaneous unidirectional countertransport of sodium ions.

When the intervesicular N-methylquinolinium is excluded by gel-chromatographic filtration, a strong outwardly-directed concentration gradient is generated. By contrast, the gel-filtration employs the

same eluent buffer--0.0100 M NaH_2PO_4, at pH = 7.45--in which the assemblies are prepared, therefore the concentrations of sodium ions found within the intra- and intervesicular volumes are equal, about 0.0200 M. It follows that the thermodynamic driving force for extrusion of N-methylquinolinium is much larger than that for the transport of sodium ions.

Furthermore, the electrostatically light N-methylquinolinium is more soluble in the membranogenic mosaic than the strongly hydrophilic, electrostatically dense, sodium ions. Moreover, the skeleton of N-methylquinolinium is flat, to facilitate intercalation, and swift vertical diffusion past adjacent hydrocarbon chains in the anisotropic membrane; certainly experiencing much less viscous drag than a spherical particle.

Henceforth, the outward current of heterocyclic cations is always slightly ahead of the slower, electroneutralizing, counterflow of sodium ions. At time zero, at 45°C, it clearly follows that the distribution of the fluorophore between the endo- and exovesicular surfaces is strongly perturbed, and relaxes back to its equilibrium position as the permeation evolves

$$Q_e^+ \overset{\text{fast}}{\rightleftharpoons} Q_x^+ \tag{27}$$

at a rate controlled by the slower antiparallel flow of sodium ions,

$$Na_x^+ \xrightarrow{\text{slow}} Na_e^+ \tag{28}$$

Finally, at equilibrium, N-methylquinolinium diffuses on to the exovesicular surface as fast as it diffuses back into the vesicles, such that, in the absence of net transport of the fluorophore, the sodium ion countercurrent ceases. Thus, the fluorescent cation functions as an optical indicator that measures the rate of influx, in the absence of efflux, of sodium ions at 45°C, across the membrane of dihexadecylphosphate vesicles.

d. The first order rate constant for translocation of sodium ions is dispersive.

The vertical diffusion of strongly hydrophilic metal ions, such as sodium, past the membrane of vesicles, deviates from a single exponential course as the diffusion time increases.[38,73] To circumvent this difficulty, it is useful to measure the permeability coefficient during an early stage, while the diffusion obeys a monoexponential rate law. Yet the exponential kinetic analysis produced in this manner is complicated by the fact that the permeation is quite slow, and therefore, the extent of diffusion, or number of half-lives, on which the process obeys an exponential rate, is very limited. Thus, to establish an appropriate comparison with the permeability constants reported in the literature, it is important that the rate processes in the suspension of interest and in the reference system extend over similar periods of time.

Likewise, the extrusion of N-methylquinolinum from DHP vesicles, at pH = 7.45 and 45°C, deviates from a single exponential rate profile. As the length of the permeation increases, the membranogenic diffusion of the fluorophore becomes progressively slower and

multiexponential. Therefore, to demonstrate that the rate of discharge of N-methylquinolinium on the exovesicular surface is controlled by the rate of countertransport of sodium ions, it is necessary to measure the rate of translocation of the heterocyclic cations during the early stage of the diffusion, while the process follows approximately a single exponential kinetic law, and to compare the rate coefficient with that observed for sodium ions crossing dipalmitoyl lecithin bilayers, over a comparable translocation time, near 45°C.

In this respect, it is useful to resolve the transmembrane diffusion of N-methylquinolinium in terms of a fast and a slow first order process, by applying a biexponential equation to the evolution of the permeation. For example, the data in Table 17 correspond to the biexponential permeation of N-methylquinolinium across gel-chromatographed DHP vesicles, suspended in 0.0100 M NaH_2PO_4, at pH = 7.45. The rapidly decaying term yields the rate constant, k_f, at 9.79×10^{-3} min^{-1}, while the slowly decaying component generates the rate coefficient, k_s, about 6.17×10^{-4} min^{-1}. Further, observe that the temporal evolution of the exponential terms is widely different. Thus, the rate constant corresponding to the slower decay may be used in an approximate comparison with the monoexponential rate coefficients reported in the literature, when the transmembrane permeation has been followed for a prolonged period of time. Conversely, the rate constant for the faster decay may be used in an approximate comparison with the monoexponential coefficients reported in the literature, when the translocation has been followed over a brief period of time.

Table 17. Biexponential Simulation of the Rate of Exovesicular Discharge of N-methylquinolinium from Gel-chromatographed Dihexadecylphosphate Vesicles, at 45°C and pH = 7.45,[a] Measured using Continuous Emission Fluorometry.[b]

Time, min	Observed C/Co	Calculated C/Co	$10^{-3} \cdot k_f$, min^{-1}	A_f, Percent	$10^{-4} \cdot k_s$, min^{-1}	A_s, Percent	rms
0	1.000	0.992	9.79 ± 4.00	13 ± 2	6.17 ± 0.26	87 ± 2	0.0168
15	0.968	0.966					
30	0.938	0.943					
45	0.911	0.923					
60	0.895	0.904					
90	0.881	0.870					
120	0.849	0.842					
180	0.792	0.795					
240	0.765	0.757					
450	0.655	0.656					
630	0.575	0.586					
790	0.512	0.531					
1500	0.356	0.342					
1860	0.310	0.274					
2220	0.237	0.220					
2970	0.121	0.138					
3300	0.110	0.112					
3600	0.0485	0.0938					

[a]The buffer is made up of 0.0100 M NaH_2PO_4; the conditions for the gel-chromatographic filtration are as described in Table 2.

[b]To compensate for the fluctuations in the output of the source, the fluorescence is measured as the ratio between the intensity from the gel-chromatographed suspension and the intensity from a reference solution of N-methylquinolinium in the pure phosphate buffer.

It should be pointed out, however, that the two rate constants extracted from the biexponential simulation are not simply related to the bimodal distribution in the diameter of the vesicles. It is easy to demonstrate this fact from a calculation of the range, or dispersion, in the rate constant expected from the radial distribution of the organizates. Accordingly, from equation (24), the ratio between the rate constant, k_{sm}, of the small 400 angstrom assemblies, and the rate constant, k_{ℓ}, of the large 1400 angstrom vesicles, is given by

$$\frac{k_{sm}}{k_{\ell}} = \frac{r_{\ell}}{r_{sm}} = \frac{650 \times 10^{-8} \text{ cm}}{150 \times 10^{-8} \text{ cm}} = 4.33$$

where r_{ℓ} and r_{sm} are the respective component radii. Clearly, the rate constant for permeation across the membrane of the smaller vesicles is only four times faster relative to their larger counterparts at 1400 angstroms. On the other hand, the biexponential simulation indicates that the rapidly decaying component has a rate constant, k_f, which is sixteen times faster than the rate coefficient, of the slower decay. It follows, therefore, that the dispersion in the rate constant of permeation is not entirely due to the radial heterogeneity of the gel-filtered assemblies.

Now then, Table 18 contains the temporal evolution of the exponential components in the bimodal simulation of the diffusion of N-methylquinolinium across the membrane of DHP vesicles, at 45°C and pH = 7.45. At time zero the more transient term contributes about 13% to the total fluorescence intensity. On the other hand, after 180 minutes at 45°C, the rapidly decaying component contributes nearly 3%

Table 18. The Evolution of the Exponential Components in the Bimodal Rate of Discharge of N-methylquinolinium from Gel-chromatographed DHP Vesicles, at 45°C and pH = 7.45,[a] Measured using Continuous Emission Fluorometry.[b]

Time, min	Observed C/Co	Calculated C/Co	Faster Component[c] $0.130 \exp(-k_f t)$	Slower Component[d] $0.870 \exp(-k_s t)$	rms
0	1.000	1.000	0.130	0.870	0.0168
15	0.968	0.966	0.112	0.854	
30	0.938	0.943	0.0969	0.846	
45	0.911	0.923	0.0836	0.839	
60	0.895	0.904	0.0722	0.831	
90	0.881	0.870	0.0539	0.816	
120	0.849	0.842	0.0401	0.802	
180	0.792	0.795	0.0223	0.773	
240	0.765	0.757	0.0124	0.745	
450	0.655	0.656	-	0.654	
630	0.577	0.586	-	0.586	
790	0.512	0.531	-	0.531	
1500	0.356	0.342	-	0.342	
1860	0.310	0.274	-	0.274	
2220	0.237	0.220	-	0.220	
2970	0.121	0.138	-	0.138	
3300	0.110	0.113	-	0.113	
3600	0.0485	0.0938	-	0.0938	

[a]The buffer is made up of 0.0100 M NaH_2PO_4; the conditions for the gel-chromatographic filtration are as described in Table 2.

[b]To compensate for the fluctuations in the output of the lamp, the fluorescence is measured as the ratio between the intensity from the gel-chromatographed suspension and the intensity from a reference solution of N-methylquinolinium in the pure phosphate buffer.

[c]The value of k_f is 9.79×10^{-3} min^{-1}, as given in Table 17.

[d]The value of k_s is 6.17×10^{-4} min^{-1}, as given in Table 17.

to the total fluorescence intensity--which corresponds to the inherent uncertainty in the fluorometric method. Thereby, the number of half-lives over which the faster exponential diffusion has been followed, becomes

$$\lambda_f = \frac{180 \text{ min}}{0.693/k_f} = \frac{180 \text{ min}}{0.693/1.00 \times 10^{-2} \text{ min}^{-1}} = 2.60.$$

By comparison, Tsong and El-Mashak[67] have measured the rate of influx of $^{22}_{11}Na$, in the absence of efflux, into unilamellar dipalmitoylphosphatidylcholine vesicles, in their fluid or liquid-crystal state, at 42°C and pH = 7.2. At this temperature and pH, they measured the permeability of the bilayer toward Na^+ to be about 6.0×10^{-10} cm/sec. The corresponding rate constant of permeation may be calculated, from equation (24), by taking into consideration both the internal volume and area of the DPPC liposomes. Accordingly, Tsong and El-Mashak[67] measured the diameter of their liposomes to be 940 ± 50 angstroms, and they used 2.83×10^{-10} cm^2 and 4.49×10^{-16} cm^3, respectively, as the area and volume of their assemblies; hence, the rate constant of translocation is

$$k = 6.0 \times 10^{-10} \frac{\text{cm}}{\text{sec}} \times \frac{2.83 \times 10^{-10} \text{ cm}^2}{4.49 \times 10^{-16} \text{ cm}^3} \times \frac{60 \text{ sec}}{1 \text{ min}} =$$

$$= 2.30 \times 10^{-2} \text{ min}^{-1}$$

The number of half-lives on which the preceding rate constant is based is readily calculated from the fact that Tsong[67] followed the transmembrane diffusion of $^{22}_{11}Na$ for about 60 minutes, such that

$$\lambda = \frac{60 \text{ min}}{0.693/k} = \frac{60 \text{ min}}{0.693/2.30 \times 10^{-2} \text{ min}^{-1}} = 2.00$$

Thus, it is possible to compare the rate coefficient for Na^+ influx measured by Tsong and El-Mashak[67] with that obtained from the biexponential analysis of the permeation of N-methylquinolinium, because both diffusion processes extend over a similar period of time, and their respective rate constants span about the same number of half-lives. For instance, while the influx of $^{22}_{11}Na$ extends over 60 minutes, and the rate coefficient spans 2.00 half-times, the extrusion of N-methylquinolinium spans 180 minutes, and the rate constant extends over 2.60 half-lives.

Nevertheless, it is necessary to adjust the rate coefficient measured by Tsong and El-Mashak[67] to take into consideration the radial distribution in the suspension of gel-chromatographed DHP vesicles. Accordingly, by using the permeability, P, of unilamellar dipalmitoyl lecithin vesicles near 45°C, about 6.0×10^{-10} cm/sec, and equation (24), the rate constant for Na^+ diffusion across the 1400 angstrom vesicles is calculated to be

$$k_{1400} = P\frac{A}{V} = P\frac{3}{r} = \frac{3.0 \times 6.0 \times 10^{-10} \text{ cm/sec}}{\left(\frac{1400 \times 10^{-8} \text{ cm} - 2 \cdot d}{2}\right)} \times \frac{60 \text{ sec}}{1 \text{ min}} =$$

$$= 1.7 \times 10^{-2} \text{ min}^{-1}$$

where r_i represents the corresponding inner radius, while d stands for the thickness of the hydrocarbon bilayer, about 50 angstroms. Likewise, the rate constant for Na^+ diffusion across DPPC vesicles measuring 400 angstroms in diameter, is calculated to be 7.2×10^{-2} min^{-1}.

The results from the preceding calculations are summarized in Table 19. Listed there, as well, is the rate constant obtained from the more transient term in the biexponential diffusion of the optical indicator N-methylquinolinium across the stereometrically equivalent DHP membrane, at 45°C and pH = 7.45. Certainly, the rate coefficient about 1.0×10^{-2} min^{-1}, reported by the fluorescent cation, is comparable to the values near 45°C at pH = 7.2 in the reference scale $(1.7 - 7.2)\ 10^{-2}$ min^{-1}, calculated from the data of Tsong and El-Mashak,[67] for the membranogenic diffusion of sodium ions across dipalmitoylphosphatidylcholine vesicles ranging between 400 and 1400 angstroms in diameter. To suggest that the concept is correct, whereby the exovesicular discharge of N-methylquinolinium requires the presence of a rate-limiting countercurrent of sodium ions.

The Biionic Transmembrane Potential

When solutions of two electrolytes having a common ion and dissimilar counterions are separated by a permeable membrane, the latter tend to diffuse along their antiparallel concentration gradients, and generate a dynamic electromotive force, or potential difference, as dictated by their adsorbability and corresponding

Table 19. A Comparison Between the First Order Rate Constant for Influx of Na^+ into Dipalmitoylphosphatidylcholine Vesicles and the Observed Rate Coefficient for Extrusion of N-methylquinolinium from Dihexadecylphosphate Vesicles at Neutral pH.[a]

	Temp., °C	Phase Transition Temp., °C	Headgroup Area	Alkyl Chain Length	Calculated[b] k, min^{-1} D, 1400 Å	Calculated[b] k, min^{-1} D, 400 Å	Observed[c] k, min^{-1}
DPPC	42	41.4	70 Å^2	C_{15}	1.7×10^{-2}	7.2×10^{-2}	--
DHP	45	≥ 35[d]	70 Å^2	C_{16}	--	--	1.0×10^{-2}

[a]The gel-chromatographed DPPC vesicles are suspended in 25 mM NaCl and 5 mM HEPES buffer, at pH = 7.2. By comparison, the gel-filtered DHP vesicles are suspended in 0.0100 M NaH_2PO_4, at pH = 7.45, to contain 16 mM sodium ions.

[b]Based on the work of T. Y. Tsong and E. M. El-Mashak[67] concerning the uptake of $^{22}_{11}Na$ into small unilamellar vesicles made up of DPPC. The diameter of their vesicles is 950 ± 50 angstroms, and the corresponding rate constant for sodium uptake is 0.0235 min^{-1}. As the influx of $^{22}_{11}Na$ is followed 60 minutes, the measured rate constant extends about 2.0 half-lives.

[c]The rate coefficient is taken from the rapidly decaying component in the biexponential simulation for the release of N-methylquinolinium, as explained in the text. Since the extrusion of the fluorescent cations is followed thereby about 180 minutes, the rate constant extends about 2.60 half-lives.

[d]Liquid and solid domains coexist in the bilayer of small sonicated DHP vesicles, above 35°C, as suggested by the onset of the broad phase transition observed fluorometrically by K. Kano and his coworkers.[69]

membranogenic mobilities. The potential difference is a transient one because it decays to zero when the exchange process is complete, as the ionic composition in the separate compartments becomes identical.

Furthermore, when one of the critical ions is more strongly adsorbed on the membrane and moves across the hydrophobic matrix more rapidly than the counterflowing specie, the magnitude and direction of the transmembrane voltage are determined by the more mobile ion--the compartment into which it diffuses contains a microscopic excess of charge, according to the sign on that specie.

As mentioned in the Introduction, a biionic potential across the membrane of phospholipid vesicles has been observed earlier by Papahadjopoulos,[31] while working with asymmetric assemblies made up of anionic phosphatidylserine, or phosphatidylglycerol, containing intravesicular potassium and intervesicular sodium ions, respectively, at equal concentrations. Now, the transmembrane voltage results from the diffusion potential of potassium which is ten times more mobile than the countercurrent sodium ions. Accordingly, the intravesicular compartment is not electrically balanced, as it carries a microscopic excess of negative charge throughout the permeation. In this regard, Ohki[32] has verified the sign and measured the magnitude of the biionic potential across planar asymmetric phosphatidylserine membranes containing the same concentration of potassium and sodium ions, respectively, in opposite compartments.

To illustrate the work of Ohki,[32] when one of the chambers across a planar phosphatidylserine membrane, at pH = 7.2, contains

40 millimolar NaCl, and the other compartment contains 40 millimolar KCl, there develops a transient potential difference about 60 millivolts, and such that the KCl-containing side is at the lower or more negative potential.

Consider now the N-methylquinolinium-sodium ion countertransport. The heterocyclic cation is hydrophobic, therefore it is strongly adsorbed on the endovesicular hydrophobic-hydrophilic interface. Moreover, the fluorescent cation is electrostatically light, that is, charge delocalized, hence its membranogenic solubility is high. Further, N-methylquinolinium has a flat skeleton, thus it penetrates efficiently through the anisotropic bilayer. Furthermore, the intervesicular volume in the gel-chromatographed suspension of vesicles is large, thereby the outwardly-directed concentration gradient for the extrusion of the fluorophore is strong. In conclusion, N-methylquinolinium will diffuse readily across the membranogenic boundary.

On the other hand, consider the counter-current of sodium ions. Since sodium is strongly hydrophilic and electrostatically dense, its membranogenic solubility is very small, and therefore its passage across the hydrophobic membrane is hindered strongly. Clearly, the flow of N-methylquinolinium is always slightly ahead of the antiparallel flow of sodium ions, and, consequently, the magnitude and direction of the transmembrane voltage are determined by the diffusion potential of the heterocyclic cations. Here then, at long last, is to be found the reason behind the distribution of N-methylquinolinium between the endo- and exovesicular surfaces, as described in

the preceding Chapter: the faster intrabilayer mobility of the fluorescent cation results in electrogenic translocation--the transmembrane migration of a single charge--of N-methylquinolinium, henceforth creating a large, inside-negative, membrane voltage to which the fluorescent cation responds by becoming distributed between the endo- and exovesicular surfaces.

Again, consider the facts. It is reasonable to suppose that a small number of N-methylquinolinium ions move across the hydrophobic bilayer, to pass on to the exovesicular surface, and thereafter into the main volume of the exceedingly large, intervesicular, aqueous phase. Because the number of N-methylquinolinium probes translocated electrogenically is small--an infinitesimal fraction of the number of endovesicular fluorescent cations--the net transport of the fluorophore is zero; yet a large transmembrane potential has been created. As net or measurable transport takes place, with countertransport of sodium ions, the escaping N-methylquinolinium senses the preexistent voltage drop and responds to the strong polarization of the membrane by remaining electrostatically bound to the vesicles, while becoming distributed between the endo- and exovesicular surfaces, in a manner that resembles closely the mode of operation of membrane potential probes; like the cationic cyanine dye diS-C_3-(5) mentioned in the latter part of Chapter II.

Furthermore, when the exovesicular surface becomes saturated with N-methylquinolinium, free or unoccupied binding sites are no longer available, such that the net flow of the heterocyclic indicator and, therefore, the counterflow of sodium ions, cease. Stated

differently, when N-methylquinolinium diffuses on to the exovesicular surface as fast as it diffuses back across the membrane, the sodium ion countercurrent halts. However, observe that a strong transmembrane potential remains, because the concentration of intervesicular fluorophore is negligible and, still, an infinitesimal amount of N-methylquinolinium can be readily transferred electrogenically into the main aqueous volume, as to maintain charged the static capacitance of the membrane.

It is noteworthy that some of the work released upon extrusion of N-methylquinolinium is stored, or conserved, in the form of osmotic energy, as a comparatively small sodium ion gradient--small comparatively, because sodium ions are present initially at the same concentration on both sides of the hydrophobic bilayer.

Finally, the N-methylquinolinium-sodium ion antiport represents an electrogenic exchange pump driven by the large outwardly-directed concentration gradient of the highly mobile fluorophore. The exchange is electrogenic because the flow of the fluorescent cation is slightly ahead of the antiparallel flow of sodium. In this respect, it is significant that electrogenic pumps, that is, those in which the exchange process is not electrically equivalent, are common in living cells.[74]

In contrast with the N-methylquinolinium-sodium ion antiport, the countertransport pumps in living cells are driven by a chemical reaction, for instance, the hydrolysis of adenosine triphosphate, ATP. Here, the function of the exchange is to generate, and maintain, ion concentration gradients, and thereby, electrochemical

potential differences, also known as resting or steady-state membrane potentials, which are fundamental to the proper operation of cellular processes such as respiration; its reverse, photosynthesis; the transmission of electrical impulses among axons; and, of course, vision.

Dynamics of Countertransport of Sodium Ions Across Dihexadecylphosphate Vesicles

In regard to the movement of sodium ions across unilamellar vesicles, Phillips and his coworkers[38] observed that as the length of time for the permeation increases, the release of Na^+ from phosphatidylcholine and phosphatidylserine assemblies deviates from the single exponential course dictated by equation (23). They attributed the deviation from single exponentiality to the simultaneous operation of two mechanisms for the release of sodium ions, namely, first order translocation and second order collision-induced release of Na^+, whereby small unilamellar vesicles may rupture, transiently, as they fuse to form large unilamellar assemblies.

Now, the influx of sodium ions into DHP vesicles reported by the intravesicular optical indicator N-methylquinolinium also deviates from a single exponential course, as the length of the permeation increases--for example, as noted in Table 17, the permeation may be approximately described by two exponential terms with widely different temporal evolutions; up to about 60 hours in total diffusion time. However, the departure from a purely monoexponential evolution cannot be attributed to fusion-induced release of the optical indicator. For instance, at pH = 7.45, the surface of the assemblies

is negatively charged, as to inhibit electrostatically the close intervesicular contact required to trigger coalescence. Similarly, the suspension of gel-chromatographed vesicles is extremely dilute and, consequently, interparticle collisions are not very frequent--recall the calculation concerning the concentration of 1400 and 400 angstrom assemblies given in the preceding chapter: in the case of a gel-chromatographed suspension containing mostly 1400 angstrom organizates, at 4.20×10^{-5} M DHP, the concentration of vesicles is estimated to be about 2.2×10^{-10} M; on the other hand, if the suspension contains predominantly 400 angstrom organizates, at 4.20×10^{-5} M DHP, the concentration of vesicles is estimated to be about 3.8×10^{-9} M. Certainly, interparticle collisions, a second order process, are not very frequent and, thereby, fusion-induced release of the optical indicator is unlikely, even after prolonged incubation at 45°C.

Now, to investigate further the nature of the departure from a first order rate, it is useful to consider the definition of the corresponding rate constant. From equations (24) and (25), the unimolecular rate coefficient of permeation, k, takes the form

$$k = K \frac{D}{d}\left(\frac{3}{r_i}\right) \tag{29}$$

in which K represents the partition constant for distribution of the permeant between the aqueous phase and the hydrophobic membrane; D stands for the coefficient of diffusion across the membrane proper,

while d refers to the thickness of the hydrophobic bilayer, about 50 angstroms. Finally, r_i corresponds to the inner radius of the vesicles. Equation (29) applies for both solute efflux and influx, since the value of k is independent of the direction of flow. Phillips and his associates[38] have indicated that in the case of sodium ions, the energy barrier corresponds to the transposition step from the aqueous phase into the membrane proper. As the low dielectric constant of the bilayer reduces greatly the membranogenic concentration of sodium ions, these may be considered in a state of quasi-equilibrium with respect to aqueous Na^+. Equation (29) may then be rewritten in the form

$$k = \frac{D}{d}\left(\frac{3}{r_i}\right) \exp\left(\frac{-\Delta G^{\neq}}{RT}\right) \tag{30}$$

where $\Delta G^{\neq}$ represents the free energy of activation required to transpose sodium ions from the aqueous into the membranogenic phase. Observe that equation (30) is certainly analogous to the thermodynamic version of unimolecular rate constants, which invokes a state of near equilibrium between the reactant and its activated complex.

Now then, consider equation (30). Note in particular, the factors whereby a dispersion in the value of k, the permeation rate coefficient, results. Recalling the fact that the translocation may be simulated using a biexponential sum, and given the bimodal distribution in the diameter of the gel-chromatographed vesicles, it seems like the dispersion in the rate constant may be attributed

completely to the radial heterogeneity. However, as indicated earlier, the distribution in the rate coefficient obtained from the biexponential analysis does not correspond with that calculated from the radial dispersion.

Again, from the data listed in Table 17, the permeation can be resolved in terms of a rapidly-decaying component whose rate constant, k_f, at 9.79×10^{-3} min^{-1}, is sixteen times greater than the rate constant, k_s, at 6.17×10^{-4} min^{-1}, of the slowly-decaying component. By comparison, let k_{sm} and k_{ℓ} represent the respective coefficients for translocation across the 400 and 1400 angstrom component assemblies, while r_{sm} and r_{ℓ} stand for the corresponding inner radii of the vesicles, in turn 150 and 650 angstroms; then it is clear, this time using equation (30), that

$$\frac{k_{sm}}{k_{\ell}} = \frac{r_{\ell}}{r_{sm}} = \frac{650 \times 10^{-8} \text{ cm}}{150 \times 10^{-8} \text{ cm}} = 4.33.$$

Hence, because the ratio k_{sm}/k_{ℓ} calculated from the radial dispersion is much smaller than the quotient obtained from the biexponential simulation, it follows that the predominant factor behind the dispersive first order dynamics is more fundamental than the readily apparent size heterogeneity of the gel-chromatographed assemblies.

Hereafter, the reason for the dispersive first order dynamics must be sought between the membranogenic diffusion coefficient and the free energy of activation, both found in equation (30). However,

to evaluate these factors adequately, it is necessary to illustrate the current mechanisms whereby sodium ions are considered to permeate across the hydrophobic membrane of unilamellar vesicles.

In this regard, Trauble[34] considered that successive trans-gauche rotamers along the hydrocarbon chains create structural defects, or cavities, which function as the natural carriers in the membranogenic mosaic; some of these cavities, or kinks, are sufficiently large to occlude small molecules or ions, to translocate them across the membrane. In addition, Trauble[34] has calculated the diffusion coefficient of kinks about 1.0×10^{-5} $cm^2 \cdot sec^{-1}$. This value corresponds with the typical diffusion coefficient of small molecules, such as methanol and ethanol, in aqueous solution near ambient temperature; thus, the membranogenic diffusion of kinks is a very fast process.

Papahadjopoulos and his school,[35] on the other hand, consider that transmembrane diffusion of strongly hydrophilic matter is facilitated by the presence of liquid and solid domains, or clusters, especially in the vicinity of the thermotropic phase transition. For example, in the case of sodium ions, Papahadjopoulos[35] believes that the coexistence of heterogeneous liquid and solid domains, enhances greatly the diffusion of sodium ions, through the boundaries between such regions of microscopic disorder. Accordingly, near the phase transition temperature, the coexistence of domains proliferates such that there is observed a discontinuous, local, 100-fold increase in the permeability of lipid bilayers with respect to sodium ions.

Nagle and Scott[36] have recently criticized the theories concerning the permeability of the lipid bilayer suggested by both Trauble[34] and Papahadjopoulos.[35] From their calculations, Nagle and Scott[36] indicate that while numerous, kinks are not the predominant membranogenic carriers. In respect to the domain mechanism advanced by Papahadjopoulos and his school, Nagle and Scott indicate that the lipid bilayer phase transition represents a cooperative, concerted event, which must therefore correspond to a point discontinuity with respect to the temperature; thus to preclude the coexistence of liquid and solid domains across a broad temperature range. To illustrate, Nagle and Scott[36] point out that the local maximum in the permeability of lipid bilayers towards sodium ions, extends over a temperature about 10 °K wide; which would suggest that liquid and solid clusters coexist across that range. Yet the thermotropic phase transition has a width which is typically less than one degree. It should be pointed out, however, that for small sonicated single bilayer vesicles, the phase transition may become broad as a result of limited cooperativity, likely because of a reduction in the size of the unit undergoing the reversible transition, such that the coexistence of liquid and solid domains, over a comparatively broad temperature range, is feasible; as in the case of dihexadecylphosphate vesicles in the present investigation.

Nevertheless, Nagle and Scott[36] suggested a theory of membrane permeability which invokes the presence of lateral density fluctuations in the bilayer, near the phase transition temperature. The transient lateral compression of the bilayer creates cavities

near the water-hydrocarbon interfaces such that small molecules and ions enter and become occluded into the hydrophobic bilayer, for subsequent diffusion, or translocation, across the membrane. It is significant that the lateral density fluctuations suggested by Nagle and Scott,[36] as well as the rotameric elements of disorder, or kinks, proposed by Trauble,[34] and the domain theory advanced by Papahadjopoulos,[35] all have one property in common, namely, that these models may be generalized to include a heterogeneous collection, or ensemble, of membranogenic carriers.

Consider again, the nature of the dispersion in the rate constant for transmembrane diffusion given by equation (30). Unlike the bimodal radial distribution, the dispersion of the membranogenic carriers is created thermally and, therefore, there exists a gaussian distribution of cavities whose free volumes and diffusion coefficients fluctuate randomly about the mean value. A random oscillation in the diffusion coefficient, D, in equation (30), has only the effect of increasing the standard deviation about the component rate constants, leaving their average values unaltered.

Now, since the strong dispersion in the rate constant is not determined predominantly by the radial heterogeneity of the assemblies, or by the distribution in the diffusion coefficient of the membranogenic carriers, it follows, from equation (30), that the highly dispersive dynamics is likely to be generated by a distribution in the free energy of activation for transposing sodium ions from the aqueous phase into the membranogenic mosaic. Certainly, a distribution in the free energy of activation is fundamental.

To investigate further into the mechanism whereby the distribution in $\Delta G^{\neq}$ results, it is useful to combine the definition of the first order rate constant for vertical translocation, equation (30), with the definition of the free energy of activation for transferring sodium ions from the aqueous phase into the hydrophobic bilayer, given by the Born equation (26); hence,

$$k = \frac{D}{d}\frac{3}{r_i} \quad \exp\left[-\frac{N_A e^2}{8\pi\varepsilon_o r_+ RT}\left(\frac{1}{\varepsilon_m} - \frac{1}{\varepsilon_w}\right)\right] \tag{31}$$

Clearly, from equation (31), the free energy of activation required to transfer a fully hydrated sodium ion of radius r_+ into the hydrophobic bilayer is strongly dependent upon the membranogenic dielectric constant, ε_m. Moreover, because of the exponential relationship between ε_m and the rate coefficient, k, a small fluctuation in the dielectric constant may change the value of the rate coefficient by several orders of magnitude; thus creating dispersive first order dynamics. According to this mechanism, the membranogenic carriers whose dielectric constant is near the high end of the distribution in ε_m, are more receptive to occlude migrating sodium ions, and thereby function as the more efficient carriers. Conversely, the vacancies whose dielectric constant is near the low end of the distribution in ε_m, tend to inhibit the transfer of sodium ions into the bilayer, and therefore function as the less efficient carriers.

To illustrate the use of equation (31), and to demonstrate the influence of a fluctuation in the dielectric constant ε_m on the

dynamics of translocation, consider the work of Tsong and El-Mashak,[67] listed in Table 19, which describes the influx of sodium ions across unilamellar dipalmitoylphosphatidylcholine vesicles. Since the permeation is followed for a brief period of time, about 60 minutes at 42°C, the diffusion of Na^+ obeys, approximately, first order dynamics. Thus, the corresponding rate coefficient is weighted heavily in favor of the more receptive carriers; that is, those near the high end of the distribution in ε_m, the membranogenic dielectric constant.

Now, it is possible to obtain an estimate of ε_m for the more efficient membranogenic ion-carrying cavities by using the rate constant for Na^+ diffusion across dipalmitoyl lecithin vesicles. For example, in the case of the assemblies whose outer diameter measures 1400 angstroms, the rate constant, k, is about 2.8×10^{-4} sec^{-1}, at 42°C. Thereby, using the diffusion coefficient, D, of kinks calculated by Trauble,[34] corrected to 42°C, at 1.05×10^{-9} $m^2 \cdot sec^{-1}$; the dielectric constant, ε_w, of pure water, at 80; the hydrated radius, r_+, of sodium ions,[75] at 3.6×10^{-10} m; the length, d, of the bilayer, at 50×10^{-10} m; and the inner radius of the vesicles, r_i, at 650×10^{-10} m, equation (31) yields

$$\varepsilon_m^{-1} = \varepsilon_w^{-1} - 3.80 \times 10^7 \, m^{-1} \cdot r_+ \& n \left[\frac{kd}{D} \left(\frac{r_i}{3} \right) \right] = 0.34$$

Hence, $\varepsilon_m \cong 3.0$.

A value of 3.0 as an upper limit to the dielectric constant of the membranogenic carriers is probably reasonable, since Fendler[4] has pointed out that the dielectric constant of planar lipid bilayers measures between 2 and 5. On the other hand, the calculated value of ϵ_m at 3.0, is considerably greater than the dielectric constant in a pure hydrocarbon medium, $\epsilon = 2$. This observation suggests, in turn, that at 42°C--the phase transition temperature of dipalmitoyl lecithin, water percolates the hydrocarbon bilayer, such that the intrabilayer carriers are not void but represent microscopic pockets filled with water whose internal, or local, dielectric constant varies with the aqueous content of the cavity. Moreover, since the vacancies are induced thermally, their size, aqueous content, and internal dielectric constant fluctuate randomly.

It is noteworthy that the same value of the membranogenic dielectric constant, ϵ_m, may be obtained by using the intrabilayer diffusion coefficient of water. Accordingly, from the permeability of planar lipid bilayers with respect to water, at 25°C, measured by Walter[76] as 3.4×10^{-3} cm sec^{-1}; the partition coefficient of water molecules into hexadecane, at 25°C, measured by Finkelstein[77] as 4.2×10^{-5}; and taking the length of the planar bilayer about 50 angstroms equation (25) yields the intrabilayer diffusion coefficient, D, of water molecules

$$D = \frac{3.4 \times 10^{-3} \text{ cm sec}^{-1} \cdot 50 \times 10^{-8} \text{ cm}}{4.2 \times 10^{-5}} = 4.05 \times 10^{-5} \frac{\text{cm}^2}{\text{sec}}$$

that corresponds to about 4.0 x 10^{-9} m^2 sec^{-1}. Note that this value of the diffusion coefficient is comparable with that calculated by Trauble for the transbilayer migration of kinks near ambient temperature, about 1.0 x 10^{-9} m^2 sec^{-1}, to indicate, as he suggested, that the transport of water across the membrane represents an inherent property of the hydrocarbon chains, which is regulated by their thermal fluctuations. Thus, when the membranogenic dielectric constant is recalculated, using the diffusion coefficient of water calculated above and corrected to 42°C, about 4.3 x 10^{-9} $m^2 \cdot sec^{-1}$, equation (31) yields $\varepsilon_m^{-1} = 0.36$, hence again, $\varepsilon_m \cong 3.0$.

Consider alternatively, the dielectric constant of the less receptive carriers. Because of their higher activation energy, and correspondingly smaller permeation rate constant, the contribution of these carriers to the transport of sodium ions becomes apparent only at longer times, when the rate coefficient is weighted strongly in their favor, with the result that the translocation deviates from its early monoexponential course, as it becomes progressively slower. Suppose furthermore, as suggested by the biexponential analysis, that the rate coefficient, k_1, of the more receptive carriers of dielectric constant ε_m', about 3.0, is sixteen times faster than the rate constant, k_2, of the less receptive carriers of dielectric constant ε_m''. Accordingly, ε_m'' may be calculated from equation (31), taking the hydrated radius[75] of sodium ion at 3.6 x 10^{-10} m,

$$\frac{1}{\varepsilon_m''} = \frac{1}{\varepsilon_m'} + 3.80 \times 10^{7}\ m^{-1} \cdot r_+ \ln\left(\frac{k_1}{k_2}\right) = 0.37$$

Hence, ε_m'' is 2.7. Consequently, the fluctuation in ε_m required to induce a 16-fold decrease, or reduction, in the magnitude of the rate constant is about 10%. Although the preceding calculation is useful to illustrate the strong influence of a small fluctuation in ε_m on the permeation coefficient of strongly hydrophilic matter, it should be pointed out that a binary distribution in the membranogenic dielectric constant is physically untenable because, as indicated earlier, the ion-carrying cavities are induced thermally, hence their size, aqueous content, and dielectric constant are distributed at random.

In conclusion, the dispersion in the rate constant of permeation for sodium ions may be attributed to two distinct components--one contributor is the radial heterogeneity of the DHP vesicles; however, because the rate coefficient is inversely proportional to the radius of the assemblies, a comparatively small dispersion in the magnitude of the rate constant results. On the other hand, the more fundamental contributor to the dispersion in the rate constant is the inhomogeneous free energy required to transfer a fully hydrated sodium ion from the aqueous phase into the hydrophobic bilayer. The distribution in the free energy of activation, $\Delta G^{\neq}$, is created by local, or microscopic, fluctuations in the dielectric constant, ε_m, of the membrane; especially near the thermotropic phase transition, when water molecules likely percolate the hydrocarbon bilayer. Since the rate coefficient is related exponentially to the free energy of activation, a small fluctuation in ε_m

generates a large change in the magnitude of the permeation rate constant.

It is significant that a hydrocarbon bilayer through which water molecules percolate is compatible with the theories of membranogenic permeability discussed earlier. For instance, Trauble[34] has suggested that elements of rotameric disorder, or kinks, with their fast diffusion coefficient, are responsible for the high intrabilayer permeability of water molecules, about 3.4×10^{-3} cm·sec^{-1}. Similarly, the line dislocations, or boundaries, between solid and liquid clusters in the domain model suggested by Papahadjopoulos,[35] are filled with water molecules probably, as Bangham[12] believes, arranged in a transient, ice-like configuration, to mediate the transport of strongly hydrophilic matter, such as sodium ions. Importantly, from the work of Holtzwarth[78] and that of Tsong and their associates,[67] it has been determined that domains, and the aqueous pores which form at their corresponding boundaries, grow and shrink in the time scale of milliseconds, for multilamellar assemblies, and microseconds, for unilamellar vesicles.

Although the interfacial transposition of sodium ions into the bilayer is a slow event, such that the membranogenic carriers are likely to be present in excess, it is important to compare the time required for an ion-transporting cavity to diffuse across the membrane proper--the transit time of the carriers--against the probable lifetime of the aqueous pores at the boundaries between liquid and solid clusters. Accordingly, the transit time, k^{-1}, for transmembrane movement of the ion-carrying vacancies across vesicles which have an

inner radius of 650 angstroms, and bilayer length about 50 angstroms, may be estimated, from equation (24), by using the diffusion coefficient of kinks calculated by Trauble,[34] at 1.0×10^{-5} cm^2 sec^{-1}, thus,

$$k^{-1} = \frac{50 \times 10^{-8} \text{ cm}}{1.0 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}} \times \frac{650 \times 10^{-8} \text{ cm}}{3} = 1.1 \times 10^{-7} \text{ seconds.}$$

Likewise, for the smaller assemblies whose inner radius is 150 angstroms, k^{-1} may be estimated about 2.5×10^{-8} seconds. Clearly, the transit time of mobile structural defects is likely to be small compared with the probable lifetime of the carriers which is on the microsecond to millisecond scale, to indicate that the aqueous pockets remain distinct during translocation across the membrane.

Finally, the presence of transient structural defects, or cavities, filled with water molecules, and which function as transbilayer conductors of strongly hydrophilic matter, is certainly compatible with the suggestion by Nagle and Scott[36] that lateral density fluctuations, near the phase transition temperature, open short-lived fissures on the surface of the vesicles, as to occlude small solutes, like water or hydrated ions, for their subsequent, fast, diffusion across the membrane.

The Gaussian Distribution in $\Delta G^{\neq}$.--Since the microscopic or local fluctuations in the dielectric constant are induced thermally, they are random and oscillate periodically about some mean or average value. Thus, they generate a gaussian collection, or ensemble, of fluctuations and, according to equation (31), a similar distribution with respect to the free energy of activation, $\Delta G^{\neq}$, required to

transpose sodium ions from the aqueous phase into the membranogenic mosaic. Furthermore, as the dispersion or nonexponentiality of the permeation demonstrates, the width of the distribution in $\Delta G^{\neq}$, that is, its precision constant, or standard error, is broad compared with dynamics that take place in homogeneous solution.

The quantitative description of the dynamics in a heterogeneous chemical system has been outlined by Albery and his coworkers;[39] more recently, from an equivalent perspective, by Siebrand and Wildman,[79] in terms of a gaussian distribution in respect to the free energy of activation, $\Delta G^{\neq}$. Accordingly, Albery indicates that in a gaussian collection of observables, the fraction $dN(x)/No$ of the total number of states, No, having a value of $\Delta G^{\neq}$ within x and x + dx standard deviations away from the mean free energy of activation, $\overline{\Delta G^{\neq}}$, is given by

$$\frac{dN(x)}{No} = \text{constant} \cdot \exp(-x^2)dx \tag{32}$$

in which the reduced variable x takes on the definition

$$x = \frac{\overline{\Delta G^{\neq}} - \Delta G^{\neq}}{\sigma} \tag{33}$$

where σ represents the width parameter, precision constant, or standard deviation of the distribution. Therefore, from equation (33), the dispersion or fluctuation in the free energy of activation becomes

$$\Delta G^{\neq} = \overline{\Delta G^{\neq}} - \sigma x \tag{34}$$

Furthermore, it is useful to redefine the standard deviation in units of RT; thus,

$$\sigma = \gamma \cdot RT \tag{35}$$

such that

$$\Delta G^{\neq} = \overline{\Delta G^{\neq}} - \gamma x RT. \tag{36}$$

Now, on multiplying by $(-RT)^{-1}$ and taking the antilogarithm on both sides of equation (36), the result obtains

$$\exp(-\Delta G^{\neq}/RT) = \exp(-\overline{\Delta G^{\neq}}/RT) \exp(\gamma x). \tag{37}$$

Thereby, because the term $\exp(-\Delta G^{\neq}/RT)$ is proportional to the unimolecular rate constant, it is possible to state the dispersion in k as

$$k = \bar{k} \exp(\gamma x) \tag{38}$$

where $\bar{k}$ stands for the first order rate constant corresponding to the mean activation energy; while γ represents a dimensionless parameter whose magnitude is proportional to the standard deviation of the distribution in $\Delta G^{\neq}$.

Consider now the first order decay of a unimolecular process

$$\frac{C_t}{Co} = \exp(-kt) \tag{39}$$

in which Co and C_t refer to the initial concentration of the reactant and the concentration at some later time, t, respectively. On combining equations (38) and (39) the relationship obtains

$$\frac{C_t}{Co} = \exp[-\bar{k}t \exp(\gamma x)] \tag{40}$$

to provide the temporal evolution of the first order decay according to the presence of a broad distribution in the activation energy.

Hereafter, it is useful to define a dimensionless time

$$\tau = \bar{k}t. \tag{41}$$

Therefore,

$$\frac{C_t}{Co} = \exp[-\tau \exp(\gamma x)]. \tag{42}$$

Finally, multiplying the numerator and denominator of the right hand side of the preceding equation (42) by the probability distribution, equation (32), and integrating between the limits of minus and plus infinity gives the statement derived by Albery and his coworkers[39]

$$\frac{C_t}{Co} = \pi^{-1/2} \int_{-\infty}^{+\infty} \exp(-x^2) \exp[-\tau \exp(\gamma x)]dx. \qquad (43)$$

Now equation (43) simulates the evolution of C_t/Co in terms of the gaussian probability of the component free energies of activation in the set. Integration of the preceding improper integral presents a difficult problem which requires the use of a Laplace transform followed by an iterative sum according to Simpson's rule; as outlined clearly by Albery and his searchers.[39] The result of the numerical integration, which may be performed with the assistance of an electronic abacus,[80] provides a description of the dispersive dynamics in terms of two parameters: the rate constant $\bar{k}$, whose value is determined by the mean activation energy in the set, and the width parameter γ whose magnitude is proportional to the spread and, therefore, to the heterogeneity of the distribution. Conversely, as the value of γ becomes small, the term $\exp(\gamma x) \cong 1$ in equation (43) and the rate profile of a monodisperse first-order decay is recovered, namely, equation (39).

Henceforth, the rate of influx of sodium ions across the membrane of dihexadecylphosphate vesicles, at 45°C and pH = 7.45, is treated according to relationship 43--the Albery equation. In this fashion, the representative translocation data in Table 20 are simulated in terms of a gaussian collection of activation barriers to yield the dispersive rate coefficient, $\bar{k}$, at 9.68×10^{-4} min^{-1} and the width parameter, γ, at 1.47.

Table 20. Transmembrane Diffusion of Sodium Ions[a] across Dihexadecylphosphate Vesicles, at 45°C and pH = 7.45[b], Simulated According to the Albery Equation 43.[c]

Time, min	Observed C/Co	Calculated C/Co	γ[d]	K, min^{-1}	rms
0	1.000	0.999	1.47	9.68×10^{-4}	0.0349
15	0.950	0.975			
30	0.902	0.953			
45	0.932	0.931			
60	0.877	0.912			
90	0.829	0.875			
120	0.809	0.841			
180	0.777	0.781			
300	0.691	0.685			
420	0.623	0.609			
600	0.570	0.522			
1140	0.364	0.357			
1650	0.319	0.269			
2010	0.228	0.225			
2640	0.199	0.172			
3060	0.126	0.146			
3480	0.0415	0.126			
4050	0.0735	0.105			

[a]As indicated by the rate of counterdiffusion of N-methylquinolinium.
[b]The buffer is made up of 0.0100 M NaH_2PO_4; the conditions for the gel-chromatographic filtration are as described in Table 2.
[c]The Albery equation is based on the presence of a broad gaussian distribution in the free energy of activation to translocate sodium ions.
[d]The inherent uncertainty in the gaussian width parameter gamma is ± 15%.

While the uncertainty, or width, in the mean free energy of activation is independent of the radial polydispersity of the vesicles, it should be pointed out that, in a heterogeneous suspension, the magnitude of γ depends on both the size distribution as well as on the more fundamental fluctuations in the membranogenic dielectric constant. For example, an increase in the radial dispersion raises the uncertainty in the preexponential constant of equation (30), as to spread the distribution in ℓnk; hence to increase the width parameter γ.

The stratagem to demonstrate the contribution from the radial uncertainty to the dispersity indicator, γ, is simple. It requires a comparison between the width parameter measured on incubation of the gel-chromatographed filtrate from a well-sonicated, optically transparent, preparation against the value of γ determined using the gel-filtered eluate from a briefly-sonicated, heterogeneous, preparation of vesicles. Thus, from the data in Table 21, at a total concentration of dihexadecylphosphate equal to 1.83×10^{-3} M, vesicles which are sonicated well, for 5 minutes at 65°C, at pH = 7.45, are nearly optically transparent, having a turbidance approximately 0.040 at 500 nanometers, across a 1 cm pathlength. On incubation for 66 hours at 45°C, the respective gel-chromatographed suspension yields a rate coefficient, $\bar{k}$, about 1.26×10^{-3} min^{-1} and a width parameter, γ, approximately 1.60. By comparison, at a similar concentration of dihexadecylphosphate, namely, 1.83×10^{-3} M, vesicles which are sonicated for a brief period of time, less than two minutes at 65°C, at pH = 7.45, are heterogeneous, having a turbidance about 0.400 at 500 nanometers, across the same 1 cm pathlength; on incubation of the corresponding gel-filtrate for 66 hours

Table 21. The Influence of Radial Polydispersity and Temperature on the Kinetic Parameters in the Albery Equation (43) as Applied to the Transmembrane Diffusion of Sodium Ions[a] across DHP Vesicles.

Crude Preparation[b]		Chromatographed Suspension[c]				
Sonication time, at 65°C[d]	Turbidance at 500 nm	Diffusion time, min	Temp. °C	R, min^{-1}	γ	rms
5 min	<0.0400	3980	45	1.26×10^{-3}	1.60	0.0248
<2 min	0.432	3905	45	1.15×10^{-3}	2.20	0.0421
5 min	<0.0400	3120	55	7.13×10^{-3}	2.41	0.0191

[a]As indicated by the rate of extrusion of N-methylquinolinium.
[b]3.00×10^{-3} M N-methylquinolinium perchlorate and 1.83×10^{3} M DHP, in 0.0100 M NaH_2PO_4 at pH = 7.45. The turbidance at 500 nm is recorded at 25°C, by using a Perkin-Elmer Model 330 Spectrophotometer.
[c]The conditions for the gel-chromatographic filtration are as described in Table 2. The turbidance/absorbance at the excitation wavelength, 319 nm, is maintained below 0.0600; if necessary, by isotonic dilution employing 0.0100 M NaH_2PO_4, at pH = 7.45, as the solvent.
[d]The dispersion is carried out by using an immersion, or bath, sonicator.

at 45°C, there are recovered a rate constant at 1.15×10^{-3} min^{-1}, and a dispersion parameter, γ , about 2.20. Clearly, the greater magnitude of γ, as compared with that obtained using the gel-filtrate from the optically transparent preparation, may be attributed to the increasing radial polydispersity of the more heterogeneous, briefly sonicated, suspension of vesicles. Observe, however, that the rate constant, $\bar{k}$, remains the same in both cases; to suggest that, as the dispersion becomes broader, the average value of the distribution in $\ln k$ remains the same.

Conversely, the fluctuations in the membranogenic dielectric constant, ϵ_m, are a distinctive property of the bilayer that is independent of the colloidal radius. Moreover, the oscillations in ϵ_m are thermally induced, such that they are expected to proliferate and become more random with an increase in the temperature. Consequently, at constant radial polydispersity, increasing the incubation temperature is likely to spread the fluctuations in $\Delta G^{\neq}$, raising thereby the value of γ ; especially in the vicinity of the phase transition, when the microtopography of the membrane is metastable.

For example, as noted in Table 21, when the gel-filtrate obtained from the optically clear preparation is incubated at 55°C, the magnitude of γ increases drastically from approximately 1.60 at 45°C, up to about 2.40. Since for a small, about 3%, raise in the absolute temperature of the dilute suspension of vesicles, the radial polydispersity is likely to remain constant, the greater width parameter at 55°C can be attributed to a wider, more random, distribution in the membranogenic dielectric constant, ϵ_m; and therefore in the free energy of activation to transfer sodium ions from the aqueous phase into the hydrocarbon bilayer.

Observe, in addition, that the rate constant, R, increases from 1.0×10^{-3} min^{-1} at 45°C, up to about 7.0×10^{-3} min^{-1} at 55°C. The thermal rise in R is not necessarily the expected twofold increase because, unlike a conventional dynamic system, the free energy of activation is strongly dependent on the temperature.

The Ionic Composition of the Intervesicular Medium

It is important to modulate the ionic composition of the intervesicular medium to further investigate the mechanism for translocation of sodium ions. As ever, the concentration of the assemblies may be systematically varied, at constant ionic strength, as to investigate the contribution from intervesicle coalescence with respect to the release of the optical indicator N-methylquinolinium. Similarly, the identity of the intervesicular counterions may be changed by replacing sodium with potassium cations, and thereby study the influence of the counterflowing species on the rate at which the fluorescent probe is discharged. In addition, the concentration of intervesicular sodium ions may be increased systematically to examine its influence on the translocation dynamics.

Isotonic dilutions.--The coalescence, or fusion, of vesicles is a second order interaction whose frequency is strongly dependent on the concentration of the colliding colloids. In this respect, isotonic dilutions, using the pure eluent buffer as the solvent, provide the means to vary the concentration of the vesicles to investigate its influence on the rate constant, R, and the gaussian width parameter γ.

The data in Table 22 illustrate the influence of a serial isotonic dilution on the permeation dynamics at 45°C. Observe that the magnitude of the precision constant γ remains the same, within the experimental margin, as the dilution increases. For example, the undiluted gel-filtered suspension yields, on incubation for 55 hours at 45°C, γ at 1.70; a threefold dilution generates γ about 1.50; finally, a 6-fold dilution gives a value of γ near 1.60. Therefore, the average value of γ across a sixfold isotonic dilution is 1.59 ± 0.07.

The precise determination of $\bar{K}$ in a serial isotonic dilution, however, presents a more sensitive problem because, unlike which stands for the constant width of the gaussian universe, the dispersive rate constant becomes progressively slower with increasing diffusion time. Thus, to optimize the precision with which $\bar{K}$ is measured, it is necessary that the permeation time is the same for each dilution. Moreover, the weaker signal from the more dilute suspensions must be carefully compensated for, electronically, by increasing the sensitivity of the photovoltaic detector, such that the change in the fluorescence can be detected equally well for each sample in the set; especially at longer translocation times, when the change in the emission intensity is small. The preceding conditions are fulfilled most effectively by incubating the samples simultaneously in a fluorometer equipped with a multicompartment cell holder, thermostated at 45°C. In this manner, it is possible to measure $\bar{K}$ with satisfactory precision. Certainly, as recorded in Table 22, on incubation for 55 hours at 45°C, the undiluted collection of chromatographed DHP vesicles yields $\bar{K}$ at 1.53×10^{-3} min^{-1}; the threefold diluted suspension generates $\bar{K}$ at 1.40×10^{-3} min^{-1}; finally the

Table 22. The Influence of Isotonic Dilution on the Transmembrane Diffusion of Na^+ across Dihexadecylphosphate Vesicles at 45°C and pH = 7.45.[a,b,c]

Isotonic Dilution	Diffusion time, min	$\bar{K}$, min^{-1}	γ	rms
--	3310	1.53×10^{-3}	1.70	0.0308
3-fold	3280	1.40×10^{-3}	1.50	0.0330
6-fold	3235	1.13×10^{-3}	1.58	0.0351

[a]At constant ionic strength; by using 0.0100 M NaH_2PO_4 at pH = 7.45 as the solvent. The samples are incubated simultaneously by using a multicompartment cell holder. The weaker fluorescence of the more dilute suspensions is compensated for by increasing the sensitivity of the photovoltaic detector; such that about the same fluorescence intensity is measured for each sample. In this manner, the weak increase in emission, at longer translocation times, can be detected equally well for each suspension in the set, as to improve the precision of the stretched rate coefficient.

[b]As indicated by the rate of extrusion of N-methylquinolinium.

[c]Gel-chromatographed according to the procedure described in Table 2.

sixfold diluted sample gives R at 1.13×10^{-3} min^{-1}. The average R across a sixfold isotonic dilution is, therefore, $(1.35 \pm 0.15) \times 10^{-3}$ min^{-1}. Consequently, the observation that both γ and R are independent of the concentration of vesicles suggests that coalescence events do not participate in the course of the permeation.

Exchange of intervesicular sodium with potassium ions.--A useful means to investigate the influence of the intervesicular ionic composition on the release of the fluorophore, consists of replacing the external sodium with potassium ions, at constant ionic strength.

The stratagem to exchange the intervesicular Na^+ with K^+ ions is clear. It requires that an aliquot of the symmetric sodium-containing preparation is chromatographed across a stationary phase which has been hydrated with 0.0100 M KH_2PO_4, at pH = 7.45; while using the same potassium phosphate buffer as the eluent. When treated in this manner, the resulting gel-filtered assemblies are asymmetric to the extent that they contain intravesicular sodium and intervesicular potassium ions.

On continued incubation for 60 hours at 45°C, the asymmetric DHP vesicles yield the kinetic parameters presented in Table 23. Observe that the dispersive rate constant R, at 8.16×10^{-4} min^{-1}, and the width constant γ, at 1.20, are the same within the experimental margin, as the values of R and γ included in Table 20, respectively, 1.00×10^{-3} min^{-1} and 1.50, for the reference suspension that contains sodium ions in both the internal and external compartments. The similarity between the values of R suggests that the corresponding free energies of activation to transpose sodium and potassium ions from the aqueous into the

Table 23. The Influence of Replacing the Intervesicular Sodium with Potassium Ions on the Dynamics of Vertical Translocation[a] across the Membrane of DHP Vesicles, at 45°C and pH = 7.45.[b,c]

Intervesicular M^+	Intravesicular M^+	Diffusion time, min	R, min^{-1}	γ	rms
Na^+	Na^+	4050	9.68×10^{-4}	1.47	0.0349
K^+	Na^+	3990	8.18×10^{-4}	1.20	0.0504

[a]As indicated by the rate of extrusion of N-methylquinolinium.
[b]The vesicles are prepared in 0.0100 M NaH_2PO_4 which contains 0.0200 M sodium ions. The gel-chromatographic filtration is as described in Table 2.
[c]The asymmetric suspension that contains 0.0200 M intervesicular K^+ is prepared by filtering the symmetric, sodium-containing, vesicles across Sephadex which has been hydrated in 0.0100 M KH_2PO_4, at pH = 7.45; while using the same potassium phosphate buffer, as the eluent.

membranogenic domain are the same for both cations; thereby, according to the Born equation (26), they must have comparable hydrated radii.

The data in Table 24 include the crystallographic, Stokes and hydrated radii of sodium and potassium ions. It should be pointed out, however, that the Stokes-Einstein approximation does not accurately measure the hydrated radius of small ions. As the conductometric size frequently turns out to be less than the minimum value corresponding to the crystallographic radius. For example, the hydrodynamic, or Stokes, radius of thallium(I), at 1.20 angstroms, is smaller than the crystallographic minimum, at 1.40 angstroms; furthermore, the hydrodynamic radius of tetramethylammonium, at 2.01 angstroms, is much smaller than the crystallographic limit, at 3.47 angstroms; similarly, the hydrodynamic radius of anions is commonly much less than their crystallographic size; and most disconcertingly, the Stokes radii of iodide, bromide and perchlorate, in dimethylformamide, turn out as negative numbers.[81]

In 1955, R. H. Stokes and R. A. Robinson[82] recognized these difficulties which result from the fact that water is not a continuous medium. Moreover, many ionic radii are comparable to the effective radius of a water molecule, at 1.40 angstroms. Thus, the conditions for viscous flow are not observed for small ions, whose radii are below about 5 angstroms, and therefore, the Stokes hydrodynamic size of metal ions is not an accurate measure of their hydrated radii.[75] To circumvent these limitations, Stokes and Robinson[82] suggested the use of corrected hydrodynamic radii, which start from the postulate that tetraalkylammonium ions are not hydrated, such that their size

Table 24. The Crystal, r_x, Stokes, r_s, and Hydrated, r_h, Ionic Radii[a] of Na^+, K^+, and $(CH_3)_4\overset{+}{N}$, including Their Corresponding Free Energies of Activation for Transfer into the Hydrocarbon Bilayer of Vesicles.

	r_x, Angstroms	r_s, Angstroms	r_h, Angstroms	$\Delta G^{\ddagger}$, Kcal/mole[b]
Na^+	0.95	1.84	3.58	15
K^+	1.33	1.25	3.31	16
$(CH_3)_4\overset{+}{N}$	3.47	2.05	3.67	15

[a]Based on the work of Nightingale,[75] and the references contained therein.

[b]As calculated from the Born equation (26), by using the hydrated ionic radii and a value of 3.0 for ε_m--the membranogenic dielectric constant, derived from the rate of influx of sodium ions into dipalmitoylphosphatidylcholine vesicles, at 42°C, as explained in the text.

in aqueous solution is equal to the respective crystallographic radii. They subsequently designed a calibration curve from which to obtain the hydrated radius based on the apparent hydrodynamic, or Stokes, size of an ion.

However, Nightingale[75] pointed out in 1959, that the calibration curve of Stokes and Robinson[82] is uncertain in the case of ions whose apparent hydrodynamic radius is less than 2.5 angstroms. Thereupon Nightingale, starting from the same postulate as Stokes and Robinson, namely, that the tetraalkylammonium ions are not hydrated, except tetramethylammonium, simplified the calibration curve by plotting the crystallographic radii, beginning with tetraethylammonium, as a function of the apparent hydrodynamic size, derived from conductometric measurements. By using this alternative calibration curve, the hydrated radius of any ion may be obtained from the ordinate axis, provided its apparent Stokes radius is known.

Accordingly, Table 24 contains the corresponding hydrated radii of potassium and sodium ions, derived using the procedure suggested by Nightingale; in turn, 3.3 and 3.6 angstroms. Now then, from the Born equation (26), and taking the membranogenic dielectric constant $\epsilon_m = 3.0$, as derived from the rate of influx of $^{22}_{11}Na$ across dipalmitoyl lecithin vesicles, near 45°C, it is possible to estimate $\Delta G^{\neq}$ for both sodium and potassium as approximatley 15 Kcal/mole; which explains the observation that the rate constants for influx of these ions across DHP vesicles, at 45°C and pH = 7.45, are clearly the same, as demonstrated by the values of R recorded in Table 23.

The fact that both Na^+ and K^+ diffuse across the dihexadecylphosphate membrane in their fully hydrated state is consistent with the suggestion above that water molecules percolate the hydrocarbon bilayer at 45°C. Nevertheless, at temperatures well separated from the thermotropic phase transition, the dynamics of ion translocation may be different, as a part of the hydration shell might be stripped from the ions. In this regard, Tsong and El-Mashak[67] have measured the permeability of dipalmitoylphosphatidylcholine vesicles with respect to Na^+ and Rb^+ at the phase transition, as well as at temperatures below and above its value.

At 42°C, or the phase transition temperature of DPPC vesicles, they have measured the permeabilities of the bilayer in the cases of sodium and rubidium ions at 6.2×10^{-10} cm sec^{-1} and $11. \times 10^{-10}$ cm sec^{-1}, respectively; these values are similar, considering the fact that the translocation is followed for a brief period of time, about 60 minutes. As the hydrated radii of Na^+ and Rb^+ are also similar, at 3.60 and 3.30 angstroms, respectively, from the Nightingale method,[75] the comparable permeabilities indicate that both ions diffuse across the membrane in their fully hydrated state which again is consistent with the postulate that water molecules saturate the hydrocarbon bilayer at the thermotropic phase transition.

On the other hand, at temperatures well separated from the lipid bilayer phase transition, the permeability of the membrane with respect to rubidium is observed to be about an order of magnitude greater than in the case of sodium ions. To indicate that a part of the hydration shells is stripped, likely because water molecules

percolate the bilayer to a small extent, below or above the thermotropic phase transition.

Titration with sodium ions.--Alternatively, to examine the influence of the concentration of intervesicular electrolyte on the dynamics of sodium translocation, it is useful to titrate the chromatographed DHP assemblies with increasing amounts of Na^+, followed by continuous incubation at 45°C, to determine the corresponding rate parameters $\mathcal{R}$ and γ. The stratagem is illustrated by the data in Table 25 which lists the rate parameters measured after continuous heating, at 45°C, of several suspensions that contain increasing concentrations of intervesicular Na^+. For example, after incubation for 60 hours, the rate parameters $\mathcal{R}$ and γ for the chromatographed reference suspension in 0.0100 M NaH_2PO_4 at pH = 7.45, are 1.00×10^{-3} min^{-1} and 1.50, respectively. Addition of sodium perchlorate to yield a total intervesicular concentration of Na^+ at 0.0414 M, followed by a similar incubation period of 60 hours at 45°C, yields rate parameters which are the same, within the experimental margin, as in the reference suspension, respectively, $\mathcal{R}$ at 1.17×10^{-3} min^{-1} and γ about 1.79.

By contrast, on addition of sodium perchlorate to yield a total intervesicular concentration of Na^+ at 0.0664 M, followed by incubation at the same temperature, the magnitude of the rate coefficient $\mathcal{R}$ increases drastically by an order of magnitude, up to 2.37×10^{-2} min^{-1} while γ remains the same, about 1.80. The observation that the value of γ remains unchanged, notwithstanding the strong acceleration in the rate, suggests that sodium ions are being translocated across

Table 25. The Influence of Intervesicular Sodium and Tetramethylammonium Ions on the Dynamics of Vertical Translocation[a] across the Membrane of DHP Vesicles, at 45°C and pH = 7.45.[b,c,d]

Intervesicular		Diffusion			
$[Na^+]$, M	$[(CH_3)_4\overset{+}{N}]$, M	time, min	K, min^{-1}	γ	rms
0.0164	--	4050	9.68×10^{-4}	1.47	0.0349
0.0414	--	3980	1.17×10^{-3}	1.79	0.0451
0.0664	--	570	2.37×10^{-2}	1.80	0.0206
--	0.0180	480	2.12×10^{-2}	1.59	0.0346

[a]As indicated by the rate of extrusion of N-methylquinolinium.
[b]The vesicles are prepared in 0.0100 M NaH_2PO_4, and thereby contain about 0.0200 M intravesicular sodium ions.
[c]The symmetric, sodium-containing vesicles are gel-chromatographed according to the procedure described in Table 2. Thereupon, the intervesicular concentration of sodium ions, starting about 0.0200 M, is increased gradually by adding $NaClO_4$ from a concentrated stock solution. After the suspensions are allowed to equilibrate for 5 hours at ambient temperature, they are incubated at 45°C.
[d]The asymmetric suspension that contains 0.0180 M intervesicular $(CH_3)_4\overset{+}{N}$ is prepared by filtering the symmetric, sodium-containing vesicles across Sephadex which has been hydrated in 0.0110 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$ at pH = 7.45; while using the same tetramethylammonium phosphate buffer as the eluent.

the membrane by the same mechanism which requires the presence of a gaussian distribution of aqueous intrabilayer carriers. Moreover, the drastic increase in the rate of discharge of N-methylquinolinium, and therefore in the counterdiffusion of sodium ions, at 0.0664 M intervesicular Na^+, is not due to the creation of a sodium ion gradient, because the vesicles are allowed to equilibrate for several hours, at ambient temperature, before their incubation at 45°C. Likewise, migration of sodium ions from the aqueous phase into the hydrocarbon bilayer is very slow, hence Na^+ accumulates on the external water hydrocarbon interface; as not to become depleted.

Consider, on the other hand, replacing the intervesicular sodium with tetramethylammonium ions. The exchange may be brought about by filtering an aliquot of the crude, sodium-containing, preparation across a Sephadex matrix which has been hydrated in 0.0100 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$ at pH = 7.45; while using the same tetramethylammonium phosphate buffer as the eluent. Thus, the chromatographed, asymmetric, assemblies contain 0.0200 M external tetramethylammonium and about 0.0200 M internal sodium ions, that is, the intra- and intervesicular compartments are isotonic; hence, the vesicles are not subjected to osmotic stress.

On incubation of the asymmetric vesicles at 45°C, the rate parameters recorded in Table 25 are obtained, namely, k at 2.12×10^{-2} min^{-1} and γ about 1.59. Clearly, the presence of intervesicular tetramethylammonium induces the same, strong, rate acceleration as observed in the presence of 0.0664 M intervesicular sodium ions. However, the drastic increase in the rate of discharge of the

fluorescent indicator is not the result of a difference in the membranogenic solubilities between tetramethylammonium and sodium ions, because their corresponding hydrated radii, from Table 24, are very similar, in turn, 3.70 and 3.60 angstroms. Therefore, the free energy of activation, given by the Born equation (26), for transferring these ions into a medium of very low dielectric constant, such as the hydrocarbon bilayer, is the same, about 15 Kcal/mole.

Conversely, the fact that the width constant γ remains unchanged suggests that the same mechanism of ion translocation holds which requires the presence of a gaussian distribution of aqueous intrabilayer carriers.

Electrostrictive Acceleration of Sodium Ion Countertransport

Alternatively, the fluorescence lifetime method provides knowledge with respect to the extent of permeation observed following the strong increase in the rate constant K at 45°C, in the presence of 0.0664 M intervesicular Na^+ or 0.0200 M external $(CH_3)_4\overset{+}{N}$. For example Table 26 contains the decay parameters for N-methylquinolinium occluded within dihexadecylphosphate vesicles that are suspended in the presence of 0.0664 M sodium ions. At 25°C, the intravesicular fluorophore yields a decay characterized by the longitudinal lifetime, T_ℓ, at 10.5 nanoseconds whose frequency, A_ℓ, is 26% and the transversal lifetime, T_t, at 3.57 nanoseconds, whose frequency, A_t, is approximately 74%. Thereafter, on incubation for about 10 hours at 45°C, the mole fraction, A_ℓ, of the longer relaxation constant increases up to

Table 26. The Fluorescence Decay of Aqueous[a] and Intravesicular[b] N-methylquinolinium in the Presence of 0.0664 M Na^+, at 25°C and pH = 7.45.

	Incubation time at 45°C	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^{2c}
Aqueous	--	10.6 ± 0.019	--	--	--	1.21
Intravesicular	--	10.5 ± 0.092	26	3.57 ± 0.043	74	1.11
Intravesicular	10 hr	10.7 ± 0.061	90	3.50 ± 0.76	10	1.07

[a] 1μM N-methylquinolinium in 0.0100 M NaH_2PO_4 to which is added $NaClO_4$ from a concentrated stock solution, to give 0.0664 M total sodium ion concentration.

[b] The vesicle suspension is gel-chromatographed according to the procedure described in Table 2. Thereafter, the intervesicular concentration of sodium ions, starting at 0.0164 M, is increased up to 0.0664 M by adding $NaClO_4$, from a concentrated stock solution, whereupon the vesicles are allowed to equilibrate for 5 hours at 25°C, before measuring the fluorescence decay.

[c] The lamp calibration curve, or instrument response function, is displaced one channel to the left of the sample decay curve.

90%. By contrast, when the colloids are suspended in the presence of 20 millimolar sodium ions, the frequency, A_ℓ, from Table 9, after 100 hours of incubation at 45°C, increases to approximately 65 ± 5%, which corresponds to saturation of the exovesicular surface with N-methylquinolinium ions. Consequently, the extent of discharge of N-methylquinolinium from the DHP assemblies suspended in 66 millimolar sodium ions is greater, as indicated by the corresponding value of A_ℓ at 90%, than can otherwise be accommodated by the exovesicular surface.

Likewise, consider the decay parameters recorded in Table 27 corresponding to the transient fluorescence of N-methylquinolinium occluded within dihexadecylphosphate vesicles suspended in the presence of 20 millimolar tetramethylammonium, that is, in 0.0100 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$ at pH = 7.45. Near ambient temperature, the intravesicular fluorophore yields a decay characterized by the longitudinal lifetime, T_ℓ, at 9.61 nanoseconds whose frequency, A_ℓ, is about 32%, and the transversal lifetime, T_t, about 3.74 nanoseconds, whose frequency, A_t, is approximately 68%. Thereafter, on incubation for 8 hours at 45°C, the fluorescence decay becomes monoexponential, to yield a relaxation constant at 8.62 nanoseconds, which is identical to the fluorescence lifetime of the free N-methylquinolinium, in 0.0100 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$ at pH = 7.45.

Clearly, the predominantly monoexponential decay whose relaxation constant represents that of the fluorophore in an aqueous solution of similar composition, free of vesicles, indicates that the intravesicular N-methylquinolinium is discharged completely into the

Table 27. The Fluorescence Decay of Aqueous[a] and Intravesicular[b] N-methylquinolinium in the Presence of 0.0180 M $(CH_3)_4\overset{+}{N}$ at 25°C and pH = 7.45.

	Incubation time at 45°C	τ_ℓ, ns	A_ℓ, Percent	τ_t, ns	A_t, Percent	χ^2[c]
Aqueous	--	8.66	--	--	--	1.00
Intravesicular	--	9.61	32	3.74	68	1.08
Intravesicular	8 hr	8.62	--	--	--	1.27

[a] 1μM N-methylquinolinium in 0.0100 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$.
[b] The vesicles contain 0.0180 M intervesicular tetramethylammonium and 0.0200 M intravesicular sodium ions. The asymmetry is created by filtering the symmetric sodium-containing vesicles across Sephadex which has been hydrated in 0.0110 M $(CH_3)_4\overset{+}{N}H_2PO_4^-$, at pH = 7.45; while using the same tetramethylammonium phosphate buffer as the eluent.
[c] The lamp calibration curve, or instrument response function, is displaced one channel to the left of the sample decay curve.

main bulk of the aqueous phase. In turn, the complete discharge of the fluorophore into the bulk aqueous volume suggests that external sodium ions, at 70 millimolar, or tetramethylammonium, at 20 millimolar, exchange and displace completely the fluorescent probes from the exovesicular surface. Accordingly, the lower concentration of $(CH_3)_4\overset{+}{N}$ needed to induce the rate acceleration reflects the stronger affinity of tetramethylammonium for the hydrophobic-hydrophilic binding sites found along the water-hydrocarbon interface.

Moreover, as N-methylquinolinium becomes distributed between the intra- and intervesicular volumes, the diffusion potential along its outwardly directed concentration gradient may be estimated, as follows: at a total concentration of DHP about 4.20×10^{-5} M, the intravesicular volume in a suspension that contains predominantly 1400 angstrom vesicles, is calculated, as discussed in Chapter II, to occupy 4.0×10^{-4} cm^3; on the other hand, the intervesicular volume is approximately 2.0 mL; that is, the volume of the gel-chromatographed band of vesicles. Therefore, the diffusion potential of the fluorescent cation is approximately given by the logarithmic ratio of the volumes of the separate compartments, according to the equation of Nernst,

$$\Delta U = -27.0 \text{ mV } \ln \left(\frac{2.00 \text{ cm}^3}{4.0 \times 10^{-4} \text{ cm}^3} \right) > -200 \text{ mV}$$

where the negative sign indicates that the intravesicular space carries a microscopic excess of negative charge. Likewise, in the case of a suspension that is predominantly made up of the smaller

400 angstrom assemblies, at 4.20×10^{-5} M DHP, the diffusion potential exceeds 200 millivolts, inside negative.

A large transmembrane potential creates strong electrostrictive force fields which can deform the membranogenic matrix and alter its permeability. In this respect, Parsegian[63] has pointed out that electrostrictive pressures can puncture, or pinch, a thin layer of dielectric material, such as a membrane, to create electrically induced pores that become filled with water, thereby increasing the dielectric constant of the membrane, to facilitate translocation of strongly hydrophilic matter. Certainly, Tsong and El-Mashak[67] have demonstrated that a transmembrane potential of 210 millivolts triggers the reversible dielectric breakdown of dipalmitoylphosphatidylcholine vesicles, at pH = 7.2. Thus, when DPPC liposomes loaded with $^{22}_{11}Na$ are placed between the plates of a capacitor which is suddenly discharged, to subject the colloids to an electric field that generates a transmembrane potential of 210 millivolts, the intravesicular tracer isotope is simultaneously and completely released, to indicate the dielectric breakdown of the membrane. Since the integrity of the assemblies is maintained, the dielectric puncture is transient, or reversible.

Now, in the case of the dihexadecylphosphate vesicles, the large potential difference, which results when the fluorescent cations become distributed between the intravesicular and the external aqueous compartments, can induce the dielectric rupture of the membrane. Nevertheless, the discharge of the fluorophore takes place

on a time scale of 10 hours, when the assemblies are suspended in 66 millimolar intervesicular sodium, or, alternatively, in the presence of 20 millimolar tetramethylammonium ions, at 45°C and pH = 7.45. Thus, the electrostrictive pressure probably deforms the hydrocarbon bilayer, as to stimulate the countertransport of sodium or tetramethylammonium ions, to prevent the dielectric breakdown of the membrane. Likely, the electrostrictive deformation creates additional structural defects in the bilayer, to facilitate water percolation, thereby increasing the membranogenic dielectric constant, ε_m, to lower the activation energy, $\Delta G^{\neq}$, to transpose tetramethylammonium or sodium ions into the hydrocarbon bilayer.

A similar conclusion may be reached by using a more dynamic construct. Consider henceforth, the partition of N-methylquinolinium between the endo- and exovesicular surfaces. Early in the permeation, the strong concentration gradient generates a small electrogenic current of the readily-permeating fluorescent cations, which diffuse past the hydrophobic membrane and into the main bulk of the aqueous phase. A large, inside negative, trans-bilayer potential develops. Thereupon, the remaining fluorescent cations respond to the membrane polarization by becoming distributed between the endo- and exovesicular surfaces, according to the partition

$$Q_e^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Q_x^+ \qquad (44)$$

where Q_e^+ and Q_x^+ represent, respectively, the endo- and exovesicular fluorescent adsorbates; while k_1 and k_{-1}, in turn, stand for the first order rate constants of efflux and influx. Now, in the presence of 70 millimolar intervesicular Na^+, or 20 millimolar $(CH_3)_4\overset{+}{N}$, the fluorophore is completely exchanged from the external surface, and displaced into the bulk aqueous phase; hence, $k_{-1}[Q_x^+] = 0$. Clearly, in the absence of influx of fluorescent cations, the transbilayer voltage undergoes a rapid, exponential, rise with the electrogenic, unidirectional, discharge of N-methylquinolinium. The resulting electrostrictive pressure introduces configurational stress, which deforms the hydrocarbon bilayer, to produce a drastic increase in its permeability, especially with respect to strongly hydrophilic matter, such as charge-dense ions.

Finally, the electrostrictive gating mechanism may be demonstrated by invoking probably the most important, yet seldom appreciated, property of vesicles, that is, their microcapacitance. In this regard, a unilamellar vesicle represents a spherical microscopic condensor whose insulator is made up of the hydrophobic bilayer which has a dielectric constant $\varepsilon_m = 3.0$, near the phase transition temperature; as estimated earlier from the rate coefficient for influx of Na^+ across the membrane of dipalmitoylphosphatidylcholine vesicles, at 42°C.

The statement for the capacitance, C_m, may be derived by considering the vesicles as made up of an inner, hollow, conducting sphere of radius r_i, at the center of an outer, likewise hollow, conducting sphere of radius r_o, while the space between the spheres

is occupied by the insulating hydrocarbon bilayer of dielectric constant ε_m; then, as shown by Sears, Zemansky, and Young[83]

$$C_m = 4\pi\varepsilon_o\varepsilon_m\left(\frac{r_o r_i}{r_o - r_i}\right) \tag{45}$$

in which ε_o stands for the permittivity of empty space, at 8.85×10^{-12} coulomb^2 · newton^{-1} · m^{-2}. Thus, when r_i and r_o are expressed in meters, the capacitance has units of farad, after Michael Faraday.

Consider now, the transmembrane potential arising from electrogenic translocation of a number Z of N-methylquinolinium probes. The voltage drop, V_{io}, across the membrane is related to the capacitance, C_m, of the vesicle by the equation

$$C_m = \frac{Ze}{V_{io}} \tag{46}$$

since C_m has units of farad, the electronic charge e is equal to 1.602×10^{-19} coulombs. On combining equations (45) and (46) the statement obtains

$$V_{io} = \frac{Ze}{4\pi\varepsilon_o\varepsilon_m}\left(\frac{1}{r_i} - \frac{1}{r_o}\right) \tag{47}$$

to indicate that for a unilamellar vesicle, V_{io} is directly proportional to the number Z of N-methylquinolinium ions translocated electrogenically across the hydrophobic bilayer.

Observe that only a small number of fluorescent probes are required to cross the membrane in order to generate a large, inside negative, voltage difference. Accordingly, in the absence of influx of N-methylquinolinium, that is, when the fluorophore is exchanged

and displaced completely from its exovesicular binding sites, as in the presence of 66 millimolar external sodium, or 16 millimolar tetramethylammonium, the number of fluorescent cations that must diffuse past the membrane of a 1400 angstrom organizate, into the bulk aqueous phase, to generate a trans-bilayer voltage of 200 millivolts--the threshold of dielectric rupture--may be estimated from equation (46). Now then, taking 50 angstroms as the length of the hydrocarbon bilayer,

$$Z = 0.200 \text{ volt} \times \frac{4\pi\varepsilon\sigma_F m}{e}\left(\frac{700 \times 650}{700 - 650}\right)10^{-10}\ m \cong 400.$$

Since, as explained in Chapter II, the 1400 angstrom assemblies are estimated to contain initially 76,000 fluorescent cations, the extent of permeation required to generate the strong electrostrictive pressure is very small, about 0.5%.

Similarly, in the case of the 400 angstrom assemblies, about 25 fluorescent cations must diffuse across the membrane into the bulk aqueous volume, to generate a transmembrane potential about 200 millivolts, inside negative. Again, the 400 angstrom vesicles are estimated to contain initially 4000 heterocyclic cations, therefore, the extent of permeation required to generate a strong electrostrictive pressure is also very small, about 0.6%. Consequently, as the 0.4% extent of translocation--in the case of the 1400 angstrom vesicles, and the 0.6%--for the 400 angstrom organizates, are small, it follows that large, inside negative, membrane voltages can be generated while the net transport of N-methylquinolinium is zero. Hence, in the absence of influx, electrogenic discharge of the highly mobile

fluorescent cations, is well ahead of the slower electroneutralizing countertransport of sodium, and generates rapidly a large electrostrictive pressure that deforms the membranogenic matrix and creates electrically induced pores that become filled with water, thereby increasing the membranogenic dielectric constant, ϵ_m. An increase in ϵ_m, in turn, accelerates the antiparallel flow of sodium ions, as to prevent the dielectric rupture of the vesicles.

The converse holds as well. In the presence of exovesicular N-methylquinolinium, the strong electrostrictive stress is ameliorated by the influx of the fluorescent cations, which prevents the hydrocarbon bilayer from becoming highly distorted, while maintaining its low permeability.

Discussion of Errors

In this section there are considered some sources of uncertainty that, for the sake of clarity and simplicity, are not considered in detail earlier in the chapter, some of which include: vesicle coalescence; the measurement of the fluorescence intensity at infinity in a dispersive kinetic system; as well as a comparison of the hydrophilic-hydrophobic properties of sodium, tetramethylammonium, and N-methylquinolinium ions.

The Coalescence of Vesicles

One of the most important factors that can interfere with the first order dynamics of vertical translocation, is the presence of fusion events among the liposomes. Fendler[4] has pointed out that intervesicle fusion can occur directly, whereupon colliding liposomes

merge, without releasing their intravesicular contents, to generate large unilamellar or multilamellar assemblies. Since, from equation (24), the rate constant of translocation is inversely proportional to the radius of the vesicles, the formation of larger assemblies, by direct fusion, slows down the rate of vertical translocation. Conversely, intervesicle fusion can occur by an indirect mechanism, whereby at least one of the colliding colloids is ruptured transiently, with the simultaneous release of its intravesicular contents. Indirect fusion may, therefore, accelerate the rate of transmembrane diffusion.

The fluorometric method is especially effective to circumvent the dynamic complications introduced by fusion events, because of its inherently high sensitivity which allows the use of extremely dilute suspensions of vesicles. For example, as calculated earlier, in a chromatographed band of organizates which contains predominantly 400 angstrom assemblies, at 4.20×10^{-5} M DHP, the concentration of vesicles is in the nanomolar range; and approximately tenth nanomolar when the suspension is predominantly made up of 1400 angstrom assemblies. Certainly, in such highly dilute suspensions, intercolloidal aggregation, or fusion events, are not very frequent.

Nevertheless, photon correlation spectroscopy, or dynamic turbidimetry, provides an effective tool to investigate the coalescence of vesicles in the gel-chromatographed band, especially during prolonged incubation at 45°C, because fusion events result in the formation of larger, more turbid, and radially polydisperse colloids. The stratagem, this time, requires a measurement of the light scattering profile at different incubation times, beginning with the unheated gel-filtrate.

Consider therefore, the dynamic turbidimetric data recorded in Table 28, which correspond to a suspension of chromatographed assemblies containing about 4.20×10^{-5} M dihexadecylphosphate, in 0.0100 M NaH_2PO_4 at pH = 7.45. Using the second moment or cumulant method, the effective diameter of the unheated vesicles is about 1470 angstroms, to yield the respective chi-squared at 9.10×10^{-8}. In comparison, after incubation for 70 hours at 45°C, the effective diameter remains the same, within the experimental boundary, at 1520 angstroms; although the chi-squared parameter increases to 2.49×10^{-7}.

While the preceding observations strongly suggest the absence of fusion events among the larger organizates, they do not, however, provide information regarding fusion among the smaller 400 angstrom organizates which are present, as determined from transmission electron microscopy, in the chromatographed band, because the light scattering properties of the suspension are dominated by the heavier, more turbid, 1400 angstrom organizates. In order to investigate the occurrence of intervesicle fusion among the 400 angstrom colloids, it is necessary to employ a more concentrated suspension, such that these assemblies contribute more strongly to the scattering properties of the medium. For example, in an unfiltered suspension containing 1.83×10^{-4} M DHP, the turbidance is determined by the presence of both 400 and 1400 angstrom assemblies, as demonstrated by the bimodal histogram in Figure 4.

Accordingly, the turbidimetric data in Table 29 describe the effective diameter of the vesicles in an unfiltered suspension, at 1.83×10^{-4} M DHP, as a function of the incubation time, at 45°C. In

Table 28. The Influence of Continued Incubation at 45°C on the Diameter of Dihexadecylphosphate Vesicles, as Determined by Dynamic Turbidimetry: Gel-chromatographed Suspension, 4.20×10^{-5} M DHP in 0.0100 M NaH_2PO_4 at pH = 7.45.[a,b]

Incubation time at 45°C	Diameter at 25°C	χ^2
0 hr	1470 Å	9.09×10^{-8}
70 hr	1520 Å	2.49×10^{-7}

[a]The suspension is gel-chromatographed according to the procedure given in Table 2.
[b]The turbidimetric data are calculated by using the cumulant or second moment method.

Table 29. The Influence of Continued Incubation at 45°C on the Diameter of Dihexadecylphosphate Vesicles, as Determined by Dynamic Turbidimetry: Unfiltered Suspension, 1.83×10^{-3} M DHP in 0.0100 M NaH_2PO_4 at pH = 7.45.[a,b,c]

Incubation time at 45°C	10-Fold dilution. Diameter at 25°C	χ^2	5-Fold dilution. Diameter at 25°C	χ^2
0 hr	740 Å	1.33×10^{-7}	680 Å	9.05×10^{-8}
20 hr	765 Å	2.04×10^{-7}	840 Å	9.82×10^{-8}
60 hr	780 Å	3.63×10^{-7}	1020 Å	8.58×10^{-7}

[a]Dispersed 5 minutes at 65°C, in the absence of N-methylquinolinium, using an immersion sonicator.

[b]The dilutions are isotonic, using 0.0100 M NaH_2PO_4, at pH = 7.45, as the solvent.

[c]The turbidimetric data are calculated by employing the cumulant, or second moment, method.

the unheated suspension, the effective diameter, which represents an average value weighted by the apparent frequencies of the bimodal components shown in Figure 4, stands about 740 angstroms, while the corresponding chi-squared is calculated at 1.33×10^{-7}; after incubation for 20 hours at 45°C, the effective diameter is about 765 angstroms, to yield a chi-squared constant at 2.04×10^{-7}; finally, upon incubation for 60 hours, at the same temperature, the effective diameter is 780 angstroms, to yield a chi-squared about 3.63×10^{-7}. Notwithstanding the monotonic increase in the chi-squared constant, the observation that the effective diameter is the same, within the experimental margin, across 60 hours of incubation, at 45°C, demonstrates the absence of fusion events among the component vesicles. Furthermore, observe that the total DHP concentration in this suspension, at 1.83×10^{-4} M, is approximately four times greater than in the gel-chromatographed band, whose DHP concentration is 4.20×10^{-5} M.

Now then, consider an unfiltered suspension whose concentration of DHP, at 3.66×10^{-4} M, is twofold greater than in the preceding sample, at 1.83×10^{-4} M. As noted in Table 29, in the unheated suspension the effective diameter of the colloids stands at 680 angstroms, while the chi-squared constant is 9.05×10^{-8}. On incubation for 20 hours at 45°C, the effective diameter increases outside the experimental margin, to 840 angstroms, although the chi-squared parameter remains nearly constant, at 9.80×10^{-8}. Finally, following an incubation period of 60 hours, at the same temperature, the effective diameter undergoes a drastic change, up to 1020 angstroms, and the

chi-squared indicator increases its value by an order of magnitude, to 8.60×10^{-7}. Clearly, the strong increase in both the effective diameter and in the chi-squared indicator prove the occurrence of vesicle coalescence, or fusion, to yield larger, more turbid aggregates starting about 3.66×10^{-4} M dihexadecylphosphate. Nevertheless, the concentration of surfactant at which the onset of fusion takes place is approximately nine times greater in respect to the concentration of DHP in the gel-chromatographed band, at 4.20×10^{-5} M.

Alternatively, consider the chromatographed vesicles which are suspended in the presence of 18 millimolar tetramethylammonium or 66 millimolar sodium ions. Notwithstanding the fact that the suspension is extremely dilute, the observation may be made that at the higher ionic strength in 0.0664 M intervesicular sodium ions, or in the presence of external tetramethylammonium, the degree of dissociation of the exovesicular surface is reduced, as to lower its anionic charge density, thereby facilitating fusion events by allowing the liposomes to approach more closely during collision. Furthermore, it may be suggested that the drastic rate acceleration observed in the presence of excess Na^+ or $(CH_3)_4\overset{+}{N}$ is the result of indirect fusion, in which some of the amphisomes rupture transiently, as they merge, with the simultaneous release of the intravesicular fluorophore.

It should be pointed out, however, that discharge of the fluorophore assisted by the indirect fusion mechanism represents a second order process which evolves slowly in an extremely dilute suspension of colloids; as not to induce an abrupt increase in the rate of release of the intravesicular fluorophore. Similarly,

fusion does not release the fluorophore completely. For example, in the indirect mechanism, only one of the pair of colliding assemblies is ruptured. Therefore, one-half of the intravesicular N-methylquinolinium remains occluded within a larger, hence less permeable, likely multilamellar, liposome. Moreover, the extent of release of the fluorophore is further reduced by the simultaneous occurrence of direct fusion, whereby the vesicles merge without releasing the intravesicular N-methylquinolinium, such that it merely becomes sequestered within a larger, less permeable, and possibly multilamellar organizate.

Consider, alternatively, the dynamics by which the electrostrictive acceleration in the rate of vertical transport takes place. Accordingly, in the presence of excess tetramethylammonium or sodium ions, at 45°C, the exovesicular fluorescent cations are very rapidly exchanged and displaced into the main bulk of the aqueous phase. Further, because of the high membranogenic mobility of the flat, charge-delocalized, N-methylquinolinium, a strong diffusion potential is created rapidly that deforms the bilayer to accelerate the counterflow of sodium or tetramethylammonium ions, with the simultaneous, complete, release of the fluorophore from the intravesicular compartment--well ahead of the contribution from fusion events.

In addition, it should be observed that, notwithstanding the strong acceleration in the rate of countertransport of Na^+ or $(CH_3)_4\overset{+}{N}$, the magnitude of the gaussian width parameter, γ, is the same as in the reference suspension of vesicles, in 0.0100 M NaH_2PO_4 at pH = 7.45. This result suggests that translocation of the charge-dense ions takes place by the same mechanism, which requires the presence of a gaussian set of aqueous membranogenic carriers.

The Measurement of the Fluorescence Intensity at Infinity

In a dynamic system that is made up of a distribution of exponential rate coefficients, the accurate measurement of the kinetic signal at infinity, that is, after the process has evolved for a prolonged period of time, presents a sensitive problem, because the dispersive rate deviates from a single-exponential course as it becomes progessively slower.

However, in the release of the optical indicator N-methylquinolinium from DHP vesicles suspended in 0.0100 M NaH_2PO_4, at pH = 7.45 and 45°C, the translocation ceases when the exovesicular surface becomes saturated with the fluorescent probes, that is, when all the binding sites on the external surface of the assemblies are occupied. The typical length of time required to saturate the exovesicular surface, as determined from dynamic spectrophotofluorometry, in Chapter II, is about 60 hours. Therefore, by following the increase in intensity using steady state fluorometry, for about 3600 minutes at 45°C, it is possible to derive an accurate value of the emission intensity at saturation, when discharge of N-methylquinolinium halts, at infinity.

Still, there may be observed comparatively small fluctuations in the precision of the rate constant R. As an illustration, consider the value of R, about 1.00×10^{-3} min^{-1}, recorded in Table 20, measured using the reference suspension of gel-filtered vesicles in 0.0100 M NaH_2PO_4 at pH = 7.45; by comparison, the rate constant for the repeated reference suspension, from Table 22, is 1.50×10^{-3} min^{-1}, after an incubation time of 3300 minutes, at 45°C.

Sometimes, comparatively small fluctuations in the precision of the rate coefficient result when the values of R are compared for suspensions whose fluorescence intensity is not the same. For example, in serial isotonic dilutions, the most dilute sample may turn out to yield the greatest rate coefficient in the set. However, the source of the fluctuation is instrumental, because the rate of change in the fluorescence intensity, at longer translocation times, is small and therefore its detection becomes more difficult with increasing dilution. Thus, the value of the fluorescence intensity at infinity may become dependent on the instrumental sensitivity and may be measured prematurely, to generate an apparently greater rate coefficient R.

In order to establish precise comparisons among the measured rate coefficients, especially in serial dilutions, it is necessary to perform the permeability measurements simultaneously, using a multicompartment cell holder, while adjusting the instrumental sensitivity to compensate for the weaker fluorescence of the more dilute suspensions. In this manner, about the same intensity is measured for the separate samples, such that the small increase in the fluorescence at the longer incubation times can be detected equally well for each suspension in the set.

Moreover, small fluctuations, commonly less than a factor of two, in the magnitude of R may result among repeated gel-chromatographic separations, because of dilution that may take place if the vesicle band is collected in fractions; or, alternatively, because the Sephadex matrix adsorbs dihexadecylphosphate, such that the inherent fluorescence of the chromatographed eluate may vary according to the amount of surfactant that is recovered from the column.

Conversely, the value of γ is virtually independent of the fluctuations in the magnitude of $\bar{R}$. In part because γ is proportional to the standard deviation of the mean activation energy, but also because of its inherently large natural uncertainty, at 15%, which results from the fact that precise control of the radial heterogeneity of vesicle suspensions presents a difficult technical problem. As an illustration, consider some of the characteristics of the immersion sonicator employed to prepare the vesicles. Although the sonicate is held within a vessel that is placed directly in the bath, such that the sonicator does not contaminate the preparation, the ultrasonic power density tends to decrease with age, as the internal vibrating knives become worn out; similarly, the power output fluctuates according to the extent to which the water in the sonicating tank is degassed; furthermore, the acoustic strength varies with the position and the depth to which the sonicate is immersed in the bath; finally, the intensity of the ultrasonic cavitations changes with the shape, especially the thickness of the walls, of the vessel that holds the sonicate.

The Hydrophobic-Hydrophilic Properties of Sodium, Tetramethylammonium, and N-methylquinolinium Ions

One of the contrasting properties between tetramethylammonium and sodium ions concerns the observation that the drastic increase in their rates of influx across the membrane of dihexadecylphosphate vesicles takes place at a much lower concentration of intervesicular $(CH_3)_4\overset{+}{N}$. As mentioned above, this result implies that tetramethylammonium is more efficient in displacing N-methylquinolinium from its

exovesicular residence sites, because the hydrophilic-hydrophobic binding environments found along the water-hydrocarbon reef correspond more closely with the properties of $(CH_3)_4\overset{+}{N}$.

On the other hand, within the membranogenic mosaic, the solubilities of tetramethylammonium and the fully hydrated sodium ions are the same; mostly because the intrabilayer dielectric constant is very small, and the radii of the two charge-dense particles are identical, about 3.60 angstroms.

Finally, consider the characteristic properties of N-methylquinolinium. Its vertical diffusion is thermodynamically favorable because of the presence of the large outwardly-directed concentration gradient, which is created upon gel-chromatographic exclusion of the intervesicular fluorophore. Further, its vertical translocation is fast, certainly faster than the antiparallel flux of tetramethylammonium or sodium ions, for two important reasons: the charge on N-methylquinolinium is delocalized, therefore the cation is electrostatically lighter than $(CH_3)_4\overset{+}{N}$ or Na^+, and hence much more soluble in the membranogenic domain. Moreover, N-methylquinolinium has a flat molecular skeleton, for efficient intercalation among adjacent hydrocarbon chains, and swift diffusion across the anisotropic membrane; certainly experiencing much less viscous drag than a spherical particle. For instance, cell membranes are observed to be more permeable to solutes whose shape is elongated rather than spherical--a result that may be attributed to the anisotropy of the membrane, that is, to the fact that the phospholipid molecules in the bilayer exist in a highly ordered state.[33,84]

Epilog

It is noteworthy that counterion exchange processes on the surface of vesicles have been investigated recently by Eduardo Lissi and his coworkers at the University of Santiago, in Chile, and by Whitten and his associates. For example, at the 7th International Symposium on Surfactants in Solution,[85] these researchers presented the results of their investigations concerning exchange of bromide and chloride counterions on the cationic surfaces of dioctadecyl dimethylammonium vesicles.

Similarly, with respect to the relative ability of sodium and tetramethylammonium to exchange and displace hydrophobic cations from anionic colloidal surfaces, Whitten, Bonilha and their collaborators have recently reported[86] that sodium ions are much less efficient than tetramethylammonium in exchanging and displacing methylviologen and cyanomethylpyridinium from the surfaces of dodecylsulfate micelles; because the binding affinity of tetramethylammonium for the hydrophobic-hydrophilic sites found along the water-hydrocarbon interface is stronger than in the case of sodium ions. Accordingly, in the presence of $(CH_3)_4\overset{+}{N}$, the binding capacity of sodium dodecyl sulfate micelles toward methylviologen, and cyanomethylpyridinium, is strongly depressed, even under a large excess of added Na^+.

Similarly, it should be pointed out that the work of Whitten and Bonilha[86] is consistent with the suggestion above that the drastic acceleration in the rate of discharge of N-methylquinolinium occurs at a much lower concentration of intervesicular tetramethylammonium, as compared with sodium ions, because $(CH_3)_4\overset{+}{N}$ has the stronger

affinity for the water-hydrocarbon sites found on the exovesicular surface; thereby to exchange the external N-methylquinolinium ions more efficiently.

In regard to the time-dependent fluctuations in the permeability of the hydrocarbon bilayer, it has been pointed out that they are caused by the presence of intrabilayer cavities filled with water which have variable dielectric constants. As the work of Tsong and El-Mashak[67] indicates, such cavities may be created by thermal membranogenic fluctuations or by the presence of transmembrane electric fields. The latter mechanism is illustrated by the electrostrictive acceleration in the rate of discharge of N-methylquinolinium when the dihexadecylphosphate vesicles are suspended in the presence of excess external sodium or tetramethylammonium ions. As the fluorescent cations are exchanged into the bulk aqueous phase, a strong diffusion potential is generated which hyperpolarizes the vesicles and stimulates the countertransport of Na^+. Since the width parameter, γ, remains the same, notwithstanding the drastic acceleration in the rate, it follows that the countertransport of Na^+ still requires the presence of a gaussian distribution of aqueous membranogenic carriers.

It is highly significant, in this respect, that Yoshikawa[87] and his searchers have very recently confirmed the existence of time-dependent fluctuations in the permeability of lipid bilayers exposed to transmembrane electric fields. For example, when a black lipid membrane made up of dioleoylphosphatidylcholine is exposed to 100 mV

applied potential, they detected sustained rhythmic oscillations between two states with conductivities between 50 and 500 picosiemens, respectively. Moreover, they observed that temporal gates in the membranogenic conductivity may be created in the absence of an applied electric field, by utilizing asymmetric black lipid membranes that contain equimolar solutions of KCl and NaCl, at 0.5 M respectively, in opposite compartments. In this case, the oscillations in the conductivity of the membrane result from the transient biionic potential across the black lipid membrane which arises, in turn, from the difference in the membranogenic mobilities between the counterdiffusing sodium and potassium ions. Likewise, the membrane of the dihexadecylphosphate vesicles, in the present investigation, is asymmetric to the extent that the intravesicular compartment contains N-methylquinolinium ions which are soluble and readily mobile across the membrane, while the intervesicular volume contains charge-dense sodium cations whose intrabilayer solubility is very small. Consequently, the dihexadecylphosphate membrane is polarized strongly such that the intravesicular space carries a microscopic excess of negative charge.

Yoshikawa[87] suggests that the fluctuations in the permeability of black lipid membranes made up of dioleoylphosphatidylcholine are caused by the leakage of ions through transient structural defects which develop in the hydrocarbon matrix in the presence of an electric field. Furthermore, he points out that the transport of charge-dense ions like sodium and potassium by intrabilayer defects, in the absence of channel-forming proteins, is consistent with the metamorphic model of biomembranes advanced recently by Cullis[88] and his associates.

In the metamorphic model, phospholipid molecules do not represent merely inert structural blocks, as in the fluid mosaic of Singer and Nicolson,[5] but participate rather actively in the transmembrane transport of strongly hydrophilic matter.

CHAPTER IV
EXPERIMENTAL

Instrumentation and Chemicals

The continuous emission measurements are made using a Perkin-Elmer MPF-44A or an LS-20 spectrophotometer. The fluorescence lifetimes are determined using a PRA dynamic fluorometer equipped with a nanosecond light source operated at 30 kHz, under one-half atmosphere of H_2, at 4.5 kV. Further, absorbance and/or turbidance measurements are carried out using a Perkin-Elmer 330 spectrophotometer, at 25 ± 0.1°C. On the other hand, the dynamic turbidimetric data are obtained at 25°C using a Spectraphysics diffusometer, from Brookhaven Instruments, operated at 514.5 nanometers.

The dispersion of dihexadecylphosphate is brought about using a Branson model 2200 sonicator equipped with a 3/4 gallon immersion tank. When required, the gel-chromatographed suspension of dihexadecylphosphate vesicles is incubated using a Fisher-Isotemp dry bath, model 145, thermostated at 45°C. In addition, the microvolumetric measurements are made by using Gilson, digital microliter, pipettes models P200D and P1000D, respectively. Finally, the 0.2 micrometer filters employed for ultrafiltration are acquired from Gelman Sciences Incorporated.

Dihexadecylphosphoric acid and toluenesulfonic acid are used as received from the Aldrich and Fisher Scientific Companies, respectively. Sephadex G-50 containing fine particles, 20-80 microns in size,

is acquired from Pharmacia Chemicals Inc. Other inorganic chemicals including sodium monohydrogen phosphate, sodium bromide and perchlorate, and organic solvents including carbon tetrachloride, or chloroform, are obtained from several manufacturers. Silver oxide and tetramethylammonium bromide are used as provided by the Matheson-Coleman and Bell Company, while thallium(I) nitrate is obtained from the Fisher Scientific Company. N-methylquinolinium perchlorate is prepared by metathesis of the corresponding iodide salt[89] in the presence of aqueous sodium perchlorate. Melting point measurements are uncorrected and taken with a Thomas Hoover apparatus equipped with a capillary tube as the sample holder.

The Preparation of Dihexadecylphosphate Vesicles

Into a 50 mL round bottom flask, there is placed a 5.00 mL aliquot from 9.15×10^{-3} M dihexadecylphosphoric acid in chloroform. Afterwards, the solvent is removed slowly, at 25-30°C, by using a rotary evaporator whose shaft is set to spin about 45-60 rpm. In this manner, the DHP is deposited on the walls of the flask in the form of a thin layer, as to facilitate its subsequent hydration without the formation of large DHP clusters. The thin layer of surfactant is allowed to dry under a vacuum, for about one-half hour, to remove traces of the solvent.

On drying the film, the vacuum is discharged and there is placed 6.625 mL of distilled water into the flask, followed by 375 microliters from a stock 0.250 M solution of NaH_2PO_4, at pH = 7.45. The layer of dihexadecylphosphoric acid is thereafter hydrated for 30-40 minutes at ambient pressure by slowly rotating the flask in the water

bath which is held at 70 ± 5°C. During its hydration, the thin layer of DHP is slowly stripped from the internal surface of the round-bottom flask until a dispersed, homogeneous, translucent gel obtains. The pH in the heterogeneous suspension of multilamellar vesicles may be estimated as follows: the hydrating medium contains initially 0.0134 M NaH_2PO_4, at pH = 7.45, or 8.58 x 10^{-3} M monohydrogen phosphate HPO_4^{-2}, and 4.82 x 10^{-3} M dihydrogen phosphate, $H_2PO_4^{-1}$. Similarly, the suspension contains 6.54 x 10^{-3} M dihexadecylphosphoric acid. Therefore, after acid-base reaction, to yield the sodium salt of dihexadecylphosphoric acid, the hydrating solution contains 2.04 x 10^{-3} M HPO_4^{-2} and 1.14 x 10^{-2} M $H_2PO_4^{-1}$, that is, 85% dihydrogen phosphate. Consequently, the pH in the suspension of multilamellar assemblies is approximately 6.50.

Sonication Procedure

The turbid suspension of multilamellar organizates is dispersed using an immersion sonicator thermostated at 65 ± 1°C by circulating hot water past a brief coil of thin copper tubing, 1/16 inch inside diameter, that is held in position inside the sonicating tank filled with water, up to about one inch from the top. While the temperature of the bath is equilibrating to 65 ± 1°C, the water must be degassed by turning on the sonicator for two consecutive 20-minute periods. Observe that the water in the tank must be degassed to optimize the acoustic power of the sonifier. Thereupon, the round-bottom flask containing the hydrated suspension of dihexadecylphosphate, at 70°C, is placed within the sonicator and immersed such that the level of

the water is slightly above, by about one-quarter of an inch, the level of the turbid suspension inside the round-bottom flask. After the flask and its contents are allowed to equilibrate to 65°C, for approximately 2 minutes, the suspension is irradiated with ultrasound for about 5 minutes, while swirling occasionally.

For efficient sonification, a position must be sought within the bath where a cavitation umbrella, or crest, appears on the surface of the suspension. Hereby, the sonicate may take from 2 to 4 minutes to become nearly optically transparent. After 5 minutes of sonication at 65°C, the flask is removed from the bath and the sonicate is allowed to cool down, at ambient temperature for 7 - 10 minutes, whereupon it is transferred to a 25 mL volumetric flask. Subsequently, the round-bottom flask is rinsed with several portions of a solution made up by dissolving 625 microliters of 0.250 M NaH_2PO_4, at pH = 7.45, in about 17 mL of distilled water, and the washings are transferred into the 25 mL volumetric flask that contains the sonicate. The final volume is adjusted to 25 mL by filling the flask to the mark with distilled water. Thus, in its final form, the unilamellar vesicle suspension contains 1.83×10^{-3} M DHP in 0.0100 M NaH_2PO_4 at pH = 7.45. The representative turbidance of the suspension at 300 nanometers and 25°C, measured in a Perkin-Elmer 330 spectrophotometer, across a 1 cm pathlength, using distilled water as the reference, is 0.300 ± 0.050.

The Inclusion of N-methylquinolinium within Dihexadecylphosphate Vesicles

The procedure to occlude N-methylquinolinium within DHP organized assemblies is a slightly modified version of the preceding

method. Into a 50 mL round bottom flask containing 4.57×10^{-5} moles of dihexadecylphosphoric acid adsorbed in the form of a thin layer, obtained as described above, is added 5.00 mL of a stock 1.53×10^{-2} M solution of N-methylquinolinium perchlorate, followed by 375 microliters of 0.250 M NaH_2PO_4 at pH = 7.45, and 1.625 mL of distilled water. Further, the thin layer of dihexadecylphosphoric acid is hydrated for 30 to 40 minutes at 70 ± 5°C, and sonicated for 5 minutes at 65 ± 1°C, as with the suspension of the pure dihexadecylphosphate vesicles. Hence, during sonication, the concentration of N-methylquinolinium is about 0.0109 M while that of the NaH_2PO_4 buffer is 0.0134 M, which yields 0.0220 M sodium ions, or about a twofold excess with respect to the fluorescent cations. Similarly, the concentration of dihexadecylphosphate is approximately 6.53×10^{-3} M. Afterwards, the sonicate is allowed to cool down at ambient temperature for about 7 to 10 minutes, as described earlier, followed by dilution up to 25.0 mL, using 0.00900 M NaH_2PO_4 at pH = 7.45. Therefore, in its final form, the preparation of vesicles contains 3.06×10^{-3} M N-methylquinolinium perchlorate, and 1.83×10^{-3} M DHP, in 0.0100 M NaH_2PO_4 at pH = 7.45.

The blueish suspension of vesicles is optically transparent. It should be pointed out, nevertheless, that the turbidance at 300 nanometers cannot be accurately measured because of strong interference from the N-methylquinolinium chromophore which absorbs light at 315 nanometers. Thus, in the presence of the fluorophore, the turbidance of the unchromatographed suspension may be measured at 500 nanometers, to give the representative value about 0.040 across a 1 cm pathlength, at 25°C.

On the other hand, the most direct means to increase the radial polydispersity of the suspension of vesicles requires the use of smaller sonication periods. For example, a multilamellar suspension prepared in the presence of 0.0109 M N-methylquinolinium perchlorate, as described above, and sonicated for about 2 minutes at 65°C, which on cooling for several minutes is diluted, at constant osmolarity, to 25.0 mL, yields a turbidance about 0.40 at 500 nanometers, across a 1 cm pathlength, at 25°C, as listed in Table 21.

Gel-chromatographic Exclusion of Intervesicular N-methylquinolinium

The hydrated Sephadex gel employed in the gel-chromatographic exclusion is easily prepared. Into a 250 mL Erlenmeyer flask, there is placed about 5 grams of dry Sephadex G-50 which is then hydrated at 25°C, using approximately 100 mL of 0.0100 M NaH_2PO_4 buffer solution at pH = 7.45. After swirling to disperse the Sephadex gel, the flask is stoppered and allowed to stand about 48 hours at ambient temperature. Some of the hydrated gel is transferred by using a long-necked disposable pipette into a 10.0 mL buret, having an inside diameter at 0.4 cm, which is filled with 0.0100 M NaH_2PO_4 buffer solution at pH = 7.45. The hydrated particles of Sephadex are allowed to fall down the column by gravity, with occasional elution of the mobile phase, as necessary. The final height of the stationary phase, which is held in place by a small piece of glass wool near the stopcock of the buret, is 25 cm. Thereafter, the stationary phase is eluted with 15 mL of the pure buffer, to allow the column to settle in place evenly, to give a final elution rate about 0.2 mL per minute.

The stationary phase is then loaded with 0.500 mL of the crude suspension of vesicles containing 1.83×10^{-3} M dihexadecylphosphate and 3.06×10^{-3} M N-methylquinolinium in 0.0100 M NaH_2PO_4 at pH = 7.45, and the vesicle band is eluted with 10 mL of the pure buffer. As shown by the elution pattern in Figure 6, the DHP assemblies emerge with the third and fourth mL eluate, within the void volume of the column. Moreover, the gel-chromatographed eluate is optically clear. For example, at 319 nm, the absorbance and/or turbidance of the gel-filtrate is less than 0.0600, across a 1 cm pathlength, at 25°C.

Incidentally, because the excitation maximum of N-methylquinolinium employed in the present work is 319 nm, it is important to maintain the optical density below about 0.060 at this wavelength, especially when measuring the fluorescence in the continuous emission mode, such that the intensity of the emitted light is directly proportional to the concentration of the intravesicular fluorophore.

By comparison, the absorbance and/or turbidance of the gel-filtrate obtained from the polydisperse preparation that is sonicated for 2 minutes at 65°C, is approximately 0.20 across a 1 cm pathlength, at 319 nm and 25°C. Therefore, in this case it is necessary to dilute the gel-filtrate, at constant ionic strength, to maintain the optical density and/or turbidance below about 0.0600.

Finally, the intervesicular pH may be varied by changing the composition of the buffer employed to hydrate the stationary phase, and as the eluent in the chromatographic separation. Care is needed, however, to control the concentration of sodium ions in the buffered

medium. For example, to raise the pH of 0.0100 M NaH_2PO_4 to 9.05, it is necessary to add about one equivalent of sodium hydroxide to yield a total concentration of Na^+ approximately 0.0200 M, as compared with 0.0164 M in the medium buffered at pH = 7.45.

The Recovery of Dihexadecylphosphate upon Gel-chromatographic Filtration

The quantitative measurement of the concentration of dihexadecylphosphate in the gel-chromatographed filtrate is complicated by the presence of inorganic phosphate in the eluent buffer, because most analytical procedures for determining phosphate-containing surfactants, and phospholipids, require hydrolysis in strongly acidic media, or digestion, to release their functional groups in the form of inorganic phosphate.

To circumvent the interference from the phosphate ions present in the buffer, it is necessary to apply an alternative method developed by Stewart[90] for the assay of phospholipid molecules that does not require digestion with a strong inorganic acid. The colorimetric determination is based on the formation of a 1:1 stoichiometric complex between phospholipids, such as dipalmitoylphosphatidylcholine and ammonium ferrothiocyanate. In general, the analysis by Stewart[90] requires extraction of $Fe(SCN)_3$ from an aqueous solution into an organic layer, such as chloroform, by phospholipid molecules dissolved in the nonpolar medium. The 1:1 ammonium ferrothiocyanate-phospholipid complex is strongly colored and obeys the Beer-Lambert equation. Therefore, a calibration curve may be constructed based upon the optical density and the concentration of the phospholipid. Thus,

from the calibration curve, the concentration of an unknown solution of the phospholipid in an organic solvent may be easily established, certainly without the lengthy manipulations of the acid digestion method.

The calibration curve.--Hereafter, the calibration graph is constructed by measuring the optical density of the ammonium ferrothiocyanate complex of dihexadecylphosphate for a series of stock solutions of the surfactant in carbon tetrachloride, up to approximately 3.0×10^{-4} M dihexadecylphosphate. Accordingly, the stock solution of ammonium ferrothiocyanate is prepared by placing 4.822 g of $FeNH_4(SO_4)_2 \cdot 12\ H_2O$, 3.238 g NaSCN and 1.601 g NH_4Cl in 100 mL of distilled water, at 25°C, with prolonged magnetic stirring to obtain a nearly saturated, deep brown solution.

Into a clean, 25 mL volumetric flask, there is placed 2.00 mL of a stock solution of DHP in carbon tetrachloride, and a 1.00 mL aliquot of the ammonium ferrothiocyanate reagent. On stoppering the flask, it is immersed in the bath sonicator such that the level of the water outside is slightly above the level of the sonicate inside the flask. Thereupon, the contents are sonicated for 5 minutes at 25°C, to mix closely the aqueous and the organic layers. With the volumetric flask only partially immersed in the water bath, a strong cavitation crest, or umbrella, develops on the surface of the sonicate. Note that in this case, it is not necessary to degas the water in the bath because the sonication is carried out at 25°C, such that the acoustic power of the sonicator, which varies inversely as the temperature, is sufficiently intense for the task at hand.

Following sonication, the resulting brown emulsion is allowed to stand for 2 to 3 minutes for the two phases to separate. The lower, light yellow, organic phase is then transferred into a 5.00 mL round-bottom flask, by using a long-necked disposable pipette while the aqueous layer remaining in the volumetric flask is rinsed with 1 mL of pure carbon tetrachloride. The washing is then combined with the organic layer containing the brown $Fe(SCN)_3$ - DHP complex. Subsequently, the organic solvent is removed slowly by using a rotary evaporator. The brown residue left inside the round-bottom flask is dried for several hours in a vacuum, over $MgClO_4$.

On drying, the brown solid is extracted with 2 mL of pure carbon tetrachloride for 20 minutes, while rotating the round-bottom flask over a water bath held at 50°C. After the extraction step, the light brown supernatant is transferred into a 3.00 mL volumetric flask and the remaining inorganic residue is rinsed using 0.5 mL of pure CCl_4. The washing is combined with the organic layer in the volumetric flask. Finally, the total volume of the organic layer is adjusted to 3.00 mL by filling the flask to the mark with CCl_4, thereby the optical density is measured at 422 nanometers and 25°C.

The calibration curve shown in Figure 12 has been constructed according to the preceding procedure and describes the optical density of the ammonium ferrothiocyanate-dihexadecylphosphate complex obtained using a set of stock solutions of DHP in carbon tetrachloride, up to approximately 3.0×10^{-4} M. Observe that the standard deviation of the assay is about 6%. Therefore, each determination is made in duplicate and the average optical density is recorded in the calibration curve of Figure 12.

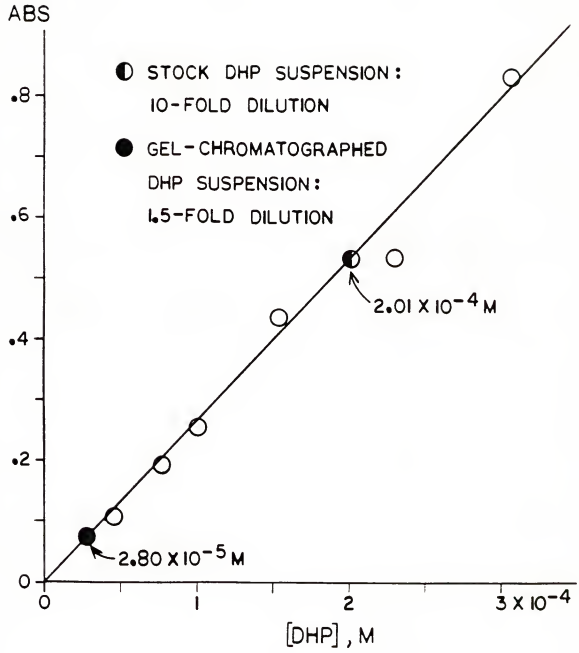


Figure 12. Absorbance of the dihexadecylphosphate · ammonium ferrothiocyanate complex in 3.00 mL of carbon tetrachloride against the concentration of DHP.

It is noteworthy that the procedure herein described represents a modification with respect to that suggested by Stewart.[90] For example, carbon tetrachloride is employed as the organic solvent, rather than chloroform, because the latter generally contains traces of ethanol which is commonly added to increase the shelf-life of $CHCl_3$. In the presence of small amounts of ethanol, the optical properties of the $Fe(SCN)_3$ - DHP complex are altered drastically, such that an optically dense, pink chromophore is obtained which does not obey the Beer-Lambert equation. Similarly, Stewart[90] suggests the use of a vortex mixer in the emulsification step. However, a bath sonicator emulsifies more efficiently because the organic and the aqueous layers are spread over a large area in the cavitation crest. Finallly, the ammonium ferrothiocyanate:dihexadecylphosphate complex is dried indirectly, without the use of inorganic hygroscopic salts, by first evaporating the carbon tetrachloride solvent, then drying the solid residue under a vacuum, followed by reextraction into carbon tetrachloride, at 50°C.

Unchromatographed Suspension of Vesicles

To illustrate the measurement of the concentration of dihexadecylphosphate in an aqueous suspension of vesicles, it is instructive to consider first the case of the stock, unchromatographed, assemblies.

1.00 mL of the stock preparation of DHP vesicles in 0.0100 M NaH_2PO_4, at pH = 7.45, is placed into a 25 mL volumetric flask and then acidified with 25 microliters of concentrated hydrochloric acid, and diluted with 2.00 mL of distilled water. The acidification step is necessary to generate the free dihexadecylphosphoric acid, in the

form of a white precipitate, which may then be extracted readily into a layer of CCl_4.

Accordingly, on addition of 5.00 mL of carbon tetrachloride, the biphasic system is sonicated with swirling, for 5 minutes at 25°C, whereupon the resulting emulsion is allowed to stand for several minutes, until the two layers separate and their interface is clearly delineated. The organic layer is transferred into a 10 mL round-bottom flask, while the aqueous layer remaining inside the volumetric flask is rinsed with about 2.0 mL of pure carbon tetrachloride. Afterwards, the washing is combined with the organic layer containing the dihexadecylphosphate extract. The organic solvent is then removed by rotary evaporation. After drying under a vacuum, the solid DHP residue is redissolved in 5 mL of CCl_4 while heating at 50°C, and rotating the flask slowly. Finally, the clear solution of dihexadecylphosphate in CCl_4 is transferred quantitatively into a 10.0 mL volumetric flask which is subsequently filled to the mark with pure CCl_4. Hence, the analyte solution of DHP in carbon tetrachloride represents a 10-fold dilution with respect to the initial 1.00 mL aliquot from the stock, unchromatographed, aqueous preparation.

Now, the concentration of dihexadecylphosphoric acid in the analyte is established by producing the $Fe(SCN)_3$ - DHP brown chromophore, according to the procedure described above. Hence, a 3.00 mL aliquot of the analyte is mixed closely, by sonication at 25°C, with 1.00 mL of the ammonium ferrothiocyanate reagent, followed by separation of the organic layer, vacuum evaporation of the solvent, drying of the solid residue under a vacuum, and reextraction into 3.00 mL

of CCl_4, to give a yellow-brown solution whose optical density at 422 nanometers and 25°C, is 0.53 ± 0.04. From the optical density, the calibration curve in Figure 12 gives the concentration of dihexadecylphosphoric acid in the analyte, about 2.01×10^{-4} M. Furthermore, because the analyte represents a 10-fold dilution, the concentration of dihexadecylphosphate in the stock aqueous preparation is 2.01×10^{-3} M which is in adequate agreement with the gravimetric concentration of DHP, namely, 1.83×10^{-3} M.

Gel-chromatographed Suspension of Vesicles

The determination of the concentration of dihexadecylphosphate in the gel-chromatographed filtrate, and thereby the percent recovery of DHP, is simple. The band containing the vesicles, that is, the combined third and fourth mL eluate from the gel-chromatographic filtration is transferred into a 25 mL volumetric flask and acidified with 50 microliters of concentrated hydrochloric acid to precipitate the dihexadecylphosphoric acid. The DHP is extracted into 3.00 mL of carbon tetrachloride with sonication for 5 minutes at 25°C. The resulting emulsion is thereafter allowed to stand for several minutes until the organic and the aqueous layers separate, whereupon the organic phase is transferred into a 5 mL round bottom flask, by using a long-necked disposable pipette, while the volumetric flask is rinsed with 1.00 mL of carbon tetrachloride and the washing combined with the organic layer containing the extracted dihexadecylphosphoric acid. Upon removal of the organic solvent using a rotary evaporator followed by drying of the residue under a vacuum, the solid is redissolved in 2.00 mL of carbon tetrachloride with heating at 50°C. The resulting

clear solution is transferred quantitatively into a 3.00 mL volumetric flask which is finally filled to the mark with carbon tetrachloride.

The concentration of dihexadecylphosphoric acid in the analyte is established, again, by producing the $Fe(SCN)_3$ - DHP brown chromophore, according to the procedure described above. Hence, the 3.00 mL extract containing the analyte is mixed closely, by sonication at 25°C, with 1.00 mL of the ammonium ferrothiocyanate reagent, followed by separation of the organic layer, vacuum evaporation of the solvent, drying of the solid residue under a vacuum, and reextraction into 3.00 mL of CCl_4 to give a yellow-brown solution whose optical density, at 422 nanometers and 25°C, from a duplicate determination, is 0.072 ± 0.015. From the calibration curve in Figure 12, the optical density gives the concentration of dihexadecylphosphoric acid in the analyte, about 2.80×10^{-5} M. Furthermore, since the volume of the analyte represents a 1.5 dilution with respect to the gel-chromatographed band, at 2.00 mL, it follows that the concentration of DHP in the gel-chromatographed band is 4.20×10^{-5} M. Similarly, the percent recovery of DHP may be calculated by comparing the amount of dihexadecylphosphate found in the chromatographed band, 84 nanomoles, against that found initially in the 0.5 mL aliquot of the unchromatographed preparation, that is, 915 nanomoles. Therefore, about 9.2% of the surfactant introduced initially into the Sephadex column is recovered in the gel-filtered eluate.

It is noteworthy that dihexadecylphosphate is found only between the third and fourth mL eluate. Moreover, preequilibration of the stationary phase with a suspension of pure dihexadecylphosphate

vesicles, free of N-methylquinolinium, increases the recovery of DHP, but lowers the resolution of the separation between inter- and intravesicular fluorophore; as the region corresponding to the aqueous fluorophore is found to overlap the band containing the intravesicular N-methylquinolinium.

Characteristic Properties of the Dihexadecylphosphate Vesicles

The DHP assemblies are characterized using three different methods which include conventional turbidimetry, dynamic turbidimetry, or photon correlation spectroscopy, and transmission electron microscopy.

Conventional Turbidimetry

Turbidimetric measurements are carried out at 25°C, utilizing an absorption spectrophotometer, across a 1 cm pathlength, while using distilled water in the reference compartment. The turbidance of pure dihexadecylphosphate vesicles, at 1.83×10^{-3} M DHP, in 0.0100 M NaH_2PO_4 at pH = 7.45, is recorded in the range between 600 and 300 nanometers to construct the corresponding Rayleigh graph of turbidance against the reciprocal fourth power of the wavelength.[42] By contrast, the suspension of gel-chromatographed assemblies, at 4.20×10^{-5} M DHP, is extremely dilute such that its turbidance in the range between 600 - 300 nanometers cannot be detected accurately by means of an absorption spectrophotometer.

Conversely, in the presence of N-methylquinolinium, the turbidance of the unchromatographed vesicles is determined at a wavelength near 500 nanometers which is well removed from the absorption band of the fluorophore at 315 nanometers.

Dynamic Turbidimetry

A more sensitive and therefore more versatile tool to measure the light scattering properties of both chromatographed, as well as unchromatographed, dihexadecylphosphate vesicles is photon correlation spectroscopy. As the laser source emits at 514.5 nanometers, it is possible to measure the light scattering properties of the organizates in the presence of N-methylquinolinium ions, without interference from the heterocyclic chromophores.

The decay of the autocorrelation function is followed past 64 hardware or memory channels. The representative sample time, or calibration, in the case of unfiltered organizates, is about 10 microseconds per channel, and the total duration of the acquisition is typically 40 seconds. On the other hand, for the gel-chromatographed vesicles, the time calibration is about 17 microseconds per channel, while the total acquisition time is typically 70 seconds. In both cases, the photovoltaic detector is set at 90 degrees with respect to the incident beam of light. Finally, each suspension of vesicles is passed through a 0.2 micrometer filter before observing the decay of the autocorrelation function at 25°C.

Likewise, in the case of the samples incubated at 45°C, the suspensions are passed through a 0.2 micrometer filter. Subsequently, the stoppered test tubes containing the vesicles are incubated at 45°C for varying periods, ranging between 20 and 70 hours, whereupon the samples are allowed to equilibrate to ambient temperature, for at least 30 minutes, before remeasuring the light scattering profile.

Transmission Electron Microscopy.--The electron micrographs are obtained by means of a JEOL transmission microscope operated at 60 kV.

A drop of the gel-chromatographed suspension is placed on a grid coated with formvar; thereafter the sample is adsorbed by using a piece of dry filter paper. Subsequently, a drop of the staining agent, 2% ammonium molybdate in ammonium hydroxide, at pH = 7.45, is placed on the surface of the grid for about 45 seconds. The drop of the staining agent is then removed by means of a piece of dry filter paper and the grid is set in place in the probe of the electron microscope for observation.

Dynamic Fluorometry

The fluorescence lifetimes are measured at 25°C unless otherwise indicated. The decay is followed past 256 channels of hardware memory while the sample time, or calibration, is about 0.195 nanoseconds per channel. The duration of the experiment, or acquisition time, is determined by the period required to accumulate 10,000 counts, at the channel of maximum intensity. Furthermore, the wavelength of the excitation light is restricted to the near ultraviolet region by using a Schott UG-11 bandpass filter with maximum transmittance at 350 nanometers. The wavelength of the emission light is restricted, in turn, to 400 nanometers by utilizing an interference filter, from Corion Corporation.

In a representative experiment, the fluorescence decay is convoluted with the lamp decay or instrument response function. In order to extract the true, or undistorted, decay it is necessary to measure the temporal pulse of the lamp itself, using the pseudo-scatterer procedure, whereby the lamp profile is reconstructed by recording a decay curve using a suspension of strongly scattering

particles, for instance, a dilute suspension prepared from dry milk. Observe, nevertheless, that the conditions under which the lamp decay curve is measured require that the wavelengths of the exciting and the scattered beams are the same. By contrast, the fluorescence decay is measured utilizing an exciting beam whose wavelength is in the near ultraviolet while the emitted light is recorded at 400 nanometers. Consequently, it is not possible to decorrelate completely the fluorescence decay, from the instrument response function, by using the pseudo-scatterer method.

Still, the lamp profile is utilized to extract some of the instrumental distortion from the true fluorescence decay by applying the least squares analysis suggested by Grinvald and Steinberg[91] using the PRA deconvolution algorithm. The method starts from the a priori assumption that the dynamics of the decay may be described by mono-, bi- or multiexponential functions. In addition, the most frequently used statistical indicator with which to investigate the fidelity of a given simulation is the chi-squared parameter. This constant has a dual function, as it measures the deviation between the experimental data and that generated by the preselected function. Hence, chi-squared measures the quality, or fidelity, of the simulation; an acceptable chi-squared value is below about 1.3, which in turn provides a means to distinguish between alternative mathematical forms of describing the decay.

The magnitude of the statistical indicator chi-squared may be optimized by using the channel displacement procedure. This method, which has been reviewed by DeSchryver and his coworkers,[57] requires

a small displacement of the lamp profile, or instrument response function, to the right or to the left of the sample decay curve, as to minimize the magnitude of chi-squared. The displacement typically represents one or two channels with respect to the position of the decay curve of the sample, and compensates for the transit-time dispersion of the fluorescence signal across the surface of the photovoltaic detector. In particular, the channel displacement method is effective to minimize the magnitude of chi-squared associated with biexponential fluorescence decays.

Finally, observe that the gel-chromatographed suspensions are optically transparent, as their turbidance is below about 0.060 at both the emission and excitation wavelengths, respectively.

Intervesicular Deactivation

The fluorescence decays in the presence of intervesicular quenchers are measured following addition of an aliquot from a concentrated stock solution of the deactivator into the recently gel-chromatographed suspension of vesicles to give, on mixing, about 0.0250 M external quencher, at 25°C.

In the case of intervesicular bromide, 33 microliters from a stock 0.775 M solution of NaBr is added into a 1.00 mL aliquot of the gel-filtered suspension of dihexadecylphosphate vesicles. Similarly, in the case of intervesicular thallium(I), 100 microliters from a stock 0.300 M solution of $T\ell NO_3$ is added into a 1.00 mL aliquot of the chromatographed DHP assemblies. The resulting concentrations of Br^- and $T\ell^+$ are 0.0250 M and 0.0273 M, respectively.

It is significant that in these experiments, particularly in the presence of 0.0250 M external Br^-, it is necessary to apply the

channel displacement method to optimize the value of the chi-squared indicator. Still, as mentioned in Chapter II, the channel displacement creates a small but systematic increase in the component lifetimes in a biexponential superposition. Therefore, it is necessary to maintain the same channel shift, while comparing sets of lifetimes from independent experiments.

Note similarly, that because the exovesicular N-methylquinolinium is accessible to intervesicular deactivators, it is useful to minimize the concentration of the fluorophore adsorbed on the outer surface of the assemblies. In this regard, it is convenient to employ the front of the vesicle-containing band, such as the first milliliter of the two-mL eluate, as the test suspension into which the thallium(I), or bromide, deactivators are added.

Intravesicular Deactivation

The influence of intravesicular collisional quenching on the fluorescence decay of internal N-methylquinolinium is investigated by occluding toluenesulfonate within the organizates, as the counterion of the fluorophore. Accordingly, N-methylquinolinium toluenesulfonate is generated by aqueous metathesis of the iodide salt of the fluorophore with one equivalent of silver toluenesulfonate.

Preparation of silver toluenesulfonate.--Into a clean 125 mL Erlenmeyer flask, there is placed 3.90 g, 16.8 millimoles, of Ag_2O and 6.40 g, 37.0 millimoles, of para-toluenesulfonic acid while the mixture is heated and stirred magnetically. The resulting clear solution is filtered and thereafter cooled in an ice bath, whereupon silver toluenesulfonate crystallizes in the form of white plates.

The crystals are collected by vacuum filtration and dried, under vacuum, in the presence of $MgClO_4$ to give about 3.65 g of silver toluenesulfonate.

Preparation of N-methylquinolinium toluenesulfonate.--Into a 50 mL Erlenmeyer flask, there is placed 2.443 g, 9.01 millimoles of N-methylquinolinium iodide, melting point 132 - 135°C, literature[92] m.p. 133°C, and 15.0 mL of distilled water. After dissolving the salt, a solution of 2.515 g, 9.01 millimoles, of silver toluenesulfonate in 10.0 mL of distilled water is added. The resulting yellow precipitate of silver iodide is removed by gravity filtration. Furthermore, the finely divided particles of AgI are excluded by passing the solution through a 0.2 micrometer filter. Thereupon, about 1.5 mL of the clear solution of N-methylquinolinium toluenesulfonate is transferred into a 50 mL volumetric flask and then diluted to the mark using distilled water. The concentration of the stock solution of N-methylquinolinium toluenesulfonate prepared in this manner is determined by absorption spectrophotometry, using dilute solutions in which the toluenesulfonate chromophore does not interfere, whereby the optical density at 315 nanometers is compared with that of a standard aqueous solution of N-methylquinolinium perchlorate. Accordingly, the concentration of the stock solution of N-methylquinolinium is determined to be 0.00986 M.

Occlusion of N-methylquinolinium toluenesulfonate.--Here the procedure to prepare the dihexadecylphosphate vesicles is the same as described above, except that a 5.00 mL aliquot from the stock 0.00986 M N-methylquinolinium toluenesulfonate is used during the sonification, instead of the 5.00 mL aliquot of 0.0153 M N-methylquinolinium

perchlorate employed routinely. Therefore, as the total volume of the sonicate is 7.00 mL, the concentration of the fluorophore during sonification is 7.04×10^{-3} M, as compared with the 0.0100 M concentration of N-methylquinolinium when the perchlorate salt is employed. Finally, the intervesicular fluorescent probes and toluenesulfonate anions are excluded by gel-chromatographic filtration using 0.0100 M NaH_2PO_4 at pH = 7.45, as the eluent.

Thermal Redistribution

To investigate the influence of the temperature on the lifetime distribution of N-methylquinolinium adsorbed on dihexadecylphosphate vesicles, the fluorescence decay of the gel-chromatographed eluate is recorded initially at 25°C, and thereupon the suspension is incubated for different periods of time by using a dry block kept at 45°C. After incubation for a specific period of time, the suspension is removed from the block and allowed to equilibrate for 30 minutes, to about 25°C, before measuring its fluorescence decay.

Dithionite reduction of exovesicular N-methylquinolinium.--After prolonged incubation of the vesicle suspension, the external N-methylquinolinium is reduced using sodium dithionite. Upon incubation for 40 to 70 hours at 45°C, the 2.0 mL suspension is removed from the heated block and allowed to equilibrate to 25°C, whereupon it is transferred into a long-necked fluorescence cell which is subsequently stoppered tightly using a small septum. The suspension is then degassed for about one hour while slowly bubbling with argon gas through a pair of hypodermic needles. In turn, the degassed stock solution of the reducing agent is prepared by placing 0.134 g of

$Na_2S_2O_4$ into a 1.00 mL volumetric flask and filling to the mark using degassed distilled water. Subsequently, the flask is stoppered tightly and argon gas bubbled slowly for about 5 minutes, to remove residual air from the flask, while stirring the solution at the same time as to dissolve the solid sodium dithionite rapidly.

On dissolving the reducing agent, 1.5 microliters of its solution is injected into the degassed vesicle suspension. Finally, the long-necked fluorescence cell is sealed by placing a drop of molten wax on the surface of the septum stopper. Note that the optical density of the suspension of vesicles after injection of the sodium dithionite aliquot, is about 0.40, at 315 nanometers and 25°C. Similarly, to facilitate the reduction of the exovesicular N-methylquinolinium, the sealed, air-tight, vesicle suspension is incubated for an additional 1-2 hr at 45°C. Then, on equilibration to 25°C, the fluorescence decay is measured. In general, the instrument response function, or lamp decay curve is displaced two channels to the left of the sample decay curve to optimize the magnitude of the chi-squared constant, below about 1.3.

Thermal Deactivation.--The fluorescence decay of N-methylquinolinium adsorbed on dihexadecylphosphate vesicles is measured in the range between 5 and 45°C to obtain the activation parameters for emission corresponding to the bimodal set of lifetimes. Typically, the thermal dependence of the lifetimes is investigated using a suspension which has been incubated for a prolonged period at 45°C, because under this condition the frequency distribution of the emissive states is at equilibrium, and hence it is independent of the temperature. The

fluorescence decays are measured sequentially in order of decreasing temperature, starting at 45 ± 0.1°C; then down to 35 ± 0.1°C, 25 ± 0.1°C , and 5 ± 0.1°C, respectively. The equilibration time before each measurement is 2.5 hours.

Osmotic Tension

The influence of osmotic stress on the fluorescence decay of N-methylquinolinium, at 25°C, is investigated by adding sodium perchlorate from a concentrated stock solution into the recently gel-chromatographed suspension of vesicles. The representative procedure requires three 2.00 mL aliquots of gel-filtered vesicles into which are added 0, 67, and 138 microliters of 0.775 M $NaClO_4$, respectively, to yield the corresponding concentrations of intervesicular sodium ions at 0.0164 M, 0.0414 M and 0.0664 M. By contrast, the intravesicular concentration of sodium ions is 0.0200 M, as determined by the amount of Na^+ present in the buffered medium in which the colloids are suspended initially.

Continuous Emission Fluorometry

The fluorescence intensity at 25°C is recorded at the emission maximum of N-methylquinolinium, at 400 nanometers, while the excitation wavelength is 319 nanometers. Observe that the optical density at the excitation wavelength is maintained below about 0.060 such that the emission intensity is directly proportional to the concentration of the luminophore.

Stern-Volmer Titrations

The presence of exovesicular N-methylquinolinium is established, as shown in Figures 9 and 10, by collisional deactivation in the presence of added bromide or thallium(I) as the external fluorescence

quenchers, in the range up to about 30 millimolar; by adding successive aliquots from a concentrated stock solution of the quencher, 0.775 M NaBr, or 0.300 M $TlNO_3$, into a 1.00 mL suspension of recently gel-chromatographed vesicles. Again, to minimize the amount of externally bound N-methylquinolinium, and thereby to reduce exchange of the exovesicular fluorophore which, as Almgren[24] indicates, induces curvature of the Stern-Volmer quenching graphs at the higher concentrations of added intervesicular deactivator, it is useful to carry out the titration using the front fraction of the band containing the vesicles, that is, the first milliliter of the two-mL eluate, which is less likely to be contaminated with external N-methylquinolinium.

Similarly, the Stern-Volmer titrations for the free N-methylquinolinium, at micromolar concentration, in 0.0100 M NaH_2PO_4 at pH = 7.45, are carried out in the continuous emission or, alternatively, in the lifetime mode, across a concentration range up to 30 millimolar in bromide or thallium(I). By comparison, deactivation of the excited state of the free N-methylquinolinium, using toluenesulfonate and measured in the lifetime mode, obeys Stern-Volmer dynamics up to a concentration about 0.0200 M in the quencher.

Dynamics of Transmembrane Diffusion

The translocation of the optical indicator N-methylquinolinium is followed continuously for 60 to 70 hours in the temperature range 45 ± 0.3°C. On transferring the 2.00 mL gel-chromatographed band into the preequilibrated fluorescence cuvette, the vesicle suspension is allowed to equilibrate thermally for 10 minutes, before the initial

fluorescence intensity is recorded. The delay period of 10 minutes is determined by measuring the time required for an equivalent volume of distilled water to equilibrate from ambient temperature up to 45 ± 0.1°C. Furthermore, a fluorescence cuvette in the cell compartment contains one micromolar N-methylquinolinium, in 0.0100 M NaH_2PO_4 at pH = 7.45, to provide a reference signal with which to correct for the temporal fluctuations in the output of the continuous emission lamp. Thus, the fluorescence measurements are expressed in the form of the ratio I/Io between the emission intensity, I, of the chromatographed eluate and that of the reference solution, Io.

When several diffusion experiments are carried out simultaneously, the gel-filtered bands are transferred in sequence into their respective preequilibrated fluorescence cuvettes, with an interval of about one-half hour between suspensions.

In general, water is circulated across a multicompartment heated cell holder in the fluorescence spectrophotometer, as to maintain a constant temperature at 45 ± 0.3°C, or better. Accordingly, to facilitate initial equilibration of the vesicle suspension from ambient temperature up to 45°C, the cell holder with the empty quartz cuvettes are allowed to equilibrate thermally overnight.

Transmembrane diffusion measurements are also carried out in the range 55 ± 0.3°C. Here again, the thermostated cell holder and its respective fluorescence cuvettes are allowed to equilibrate thermally with the surroundings overnight. On transferring the 2.00 mL gel-chromatographed eluate into its preequilibrated fluorescence cuvette,

the vesicle suspension is allowed to equilibrate thermally for 10 minutes before the initial fluorescence intensity is recorded. Once more, the delay time of 10 minutes is determined separately, by observing the time required for a similar volume of distilled water to equilibrate from ambient temperature up to 45 ± 0.1°C.

Intervesicular Ionic Composition

Isotonic dilutions.--In dilution experiments at constant ionic strength, that is, using 0.0100 M NaH_2PO_4 at pH = 7.45, as the solvent, it is necessary to employ a multicompartment cell holder, as to carry out the diffusion measurements simultaneously, across the same length of time. Furthermore, the weaker fluorescence intensity of the more dilute, gel-filtered, suspensions must be compensated for by amplifying the sensitivity of the measurement, such that about the same fluorescence intensity is recorded for each sample in the set. As indicated above, this protocol minimizes the instrumental uncertainty in the value of $\mathcal{R}$, the dispersive rate constant; in light of the fact that at the longer translocation times, the change in the emission intensity is small.

Titration with sodium ions.--To increase the intervesicular concentration of sodium ions, it is effective to add sodium perchlorate into the recently gel-chromatographed suspension of vesicles, because the perchlorate anion does not deactivate the fluorescence of N-methylquinolinium. Typically, three 2.00-mL samples are charged with 0, 67, and 138 microliters, respectively, from a stock 0.775 M solution of $NaClO_4$ in distilled water. As the initial concentration of Na^+ in each suspension is 0.0164 M, the corresponding intervesicular

concentrations of this ion, upon addition of $NaClO_4$, are 0.0164 M, 0.0414 M, and 0.0664 M. Observe that the vesicle suspensions are allowed to equilibrate at ambient temperature for 5 hours before starting the diffusion experiments at 45 ± 0.3°C. In addition, the fluorescence decay of the suspension containing 0.0664 M intervesicular Na^+ is recorded at ambient temperature both before starting the diffusion process as well as at infinity, that is, after incubation for about 10 hours at 45°C.

Exchange of intervesicular Na^+ with K^+.--The external sodium ions are readily replaced by filtering the 0.500 mL aliquot of the stock suspension of vesicles, which contains 1.83×10^{-3} M DHP in 0.0100 M NaH_2PO_4 at pH = 7.45, past a stationary phase made up of Sephadex G-50 that has been previously hydrated in 0.0100 M KH_2PO_4 at pH = 7.45, while using the same potassium phosphate buffer as the eluent. In this manner, the sodium ions initially present with the vesicles are eluted together with the external N-methylquinolinium, while the chromatographed band of asymmetric vesicles now contains intervesicular potassium and intravesicular sodium ions. The gel-chromatographed suspension is thereafter incubated at 45°C for 60 to 70 hours while the increase in the fluorescence intensity is measured in the continuous emission or in the lifetime mode, as appropriate.

Exchange of intervesicular Na^+ with $(CH_3)_4\overset{+}{N}$.--The replacement of external sodium with tetramethylammonium is brought about by passing the 0.500 mL aliquot of the stock suspension of vesicles which contains 1.83×10^{-3} M DHP in 0.0100 M NaH_2PO_4 at pH = 7.45, past

Sephadex G-50 that has been previously hydrated in 0.0100 M $(CH_3)_4NH_2PO_4$ at pH = 7.45, while using the same tetramethylammonium phosphate buffer as the eluent.

The tetramethylammonium phosphate buffer is prepared by titrating tetramethylammonium hydroxide with phosphoric acid. In turn, $(CH_3)_4NOH$ is readily obtained by metathesis of tetramethylammonium bromide with silver oxide.

Accordingly, into a 125 mL Erlenmeyer flask there is added 6.932 g, 45 millimoles, of dry tetramethylammonium bromide and 50 mL of distilled water, followed by 5.215 g, 22.5 millimoles, of silver oxide. Afterwards the heterogeneous mixture is stirred magnetically for about 2 hours at ambient temperature and then the silver bromide precipitate is removed by gravity filtration. The resulting solution of tetramethylammonium hydroxide is further passed through a 0.2 micron filter to remove the finely divided particles of AgBr which are not excluded by the gravity filtration. Subsequently, the clear solution of tetramethylammonium hydroxide is titrated using slightly more than 8 milliliters of 3 M H_3PO_4, to adjust the pH to 7.45. The solution obtained in this manner is transferred quantitatively into a 100 mL volumetric flask and diluted to the mark with distilled water to give the stock solution of tetramethylammonium phosphate, at pH = 7.45.

Thereby, the buffer solution used to hydrate the Sephadex stationary phase, and as the eluent in the gel-chromatographic separation, is prepared by taking 4.00 mL of the stock tetramethylammonium phosphate buffer and diluting it to 100 mL using distilled water. The solution that results contains 0.0180 M $(CH_3)_4\overset{+}{N}$, at pH = 7.45. By

comparison, the sodium phosphate buffer contains 0.0164 M Na^+, at the same pH.

After gel-chromatographic filtration, the asymmetric vesicles, which contain intravesicular sodium ions and intervesicular tetramethylammonium, are incubated at 45°C for about 10 hours while the increase in the fluorescence intensity is measured in the continuous emission mode, to determine the magnitude of K, the dispersive rate constant of transmembrane diffusion. In addition, the fluorescence decay of the suspension is recorded at ambient temperature both before starting the diffusion process, as well as at infinity, that is, after incubation for approximately 10 hours at 45°C.

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BIOGRAPHICAL SKETCH

Servando Muñoz was born in San Pedro sula, Honduras, on September 28, 1959. He lived there until 1976, when he graduated from LaSalle High School, after which he moved to Fort Myers, Florida, with a permanent residency status. In 1979, he graduated from Edison Community College with an Associate of Arts degree, and subsequently spent two years in Miami, Florida, obtaining his Bachelor of Science degree in chemistry from Florida International University in 1981.

His undergraduate work completed, Mr. Muñoz then came to the University of Florida in Gainesville to receive his Master of Science degree in 1985. At that time, his research work concerned the subject of hydrophobic and electrostatic binding in micellar media, which was published jointly with Dr. J. A. Zoltewicz in The Journal of Physical Chemistry, 1986, 90, 5820-25. It should be noted that Mr. Muñoz also served continuously as a Teaching Assistant (1/3 time) at the University of Florida during his entire residency while working towards the Master of Science degree.

Since 1986, with support from the Division of Sponsored Research at the University of Florida, Mr. Muñoz has worked full time on his doctoral dissertation. Part of this work has already been presented at the 39th Southeastern Regional Meeting of the American Chemical

Society, held in Orlando, Florida, in November 1987. In its final form, this research will be presented at the 198th National Meeting of the American Chemical Society, to be held in Miami, Florida, in September 1989.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John A. Zoltewicz, Chairman
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William M. Jones
Professor of Chemistry

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Robert C. Stoufer
Associate Professor of Chemistry

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Kirk S. Schanze
Assistant Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William R Kem

William R. Kem
Associate Professor of Pharmacology and Therapeutics

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1989

Dean, Graduate School